THE PATHOGENESIS OF NATURAL AND EXPERIMENTAL ARTHROPATHIES OF SHEEP

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Declaration

The experiments and composition of this thesis are, unless otherwise stated, my own work. No part of this work has been, or is being, submitted for any other degree, diploma or other qualification.

Angus Anderson (June 1995)

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ABSTRACT OF THESIS

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The pathogenesis of natural and experimental arthropathies in sheep is poorly understood. The aims of the work described in this thesis were: 1) to determine the expression of immunologically relevant molecules in synovial tissues from sheep at different stages of development from the foetus to the adult, and sheep infected with Maedi-Visna virus (MVV), a non-oncogenic retrovirus: 2) study the kinetics of inflammatory cell turnover in synovial tissues and trafficking into the draining lymph node in sheep with experimental antigen-induced arthritis (AIA)

Immunohistlogical analysis of the synovial lining and flow cytometric analysis of cells in synovial fluid (SF) from sheep of different ages showed that there was a significant increase in the proportion of cells expressing MHC class II antigens during the first few months of life. Very few lymphoid cells were found in tissues from sheep of any age.

Immunopathological studies of synovial tissues from clinically arthritic MVV-infected sheep showed that the inflammatory infiltrate was characterised by large numbers of large mononuclear cells and T lymphocytes, of which the CD8+ subset predominated over CD4+ and γδ T lymphocyte subsets. Large numbers of cells in the synovial lining expressed MHC class II and CD1 antigens. CD8+ T lymphocytes were found at a significantly higher density in some synovial tissues from MVV-infected sheep with no clinical signs of arthritis compared to tissues from control sheep. Additionally, the proportion of MHC class II-expressing cells in the subintima of these tissues was significantly higher than in tissues from control sheep.

To characterise the progression of an inflammatory arthritis in a more controlled way, an AIA was generated in a group of adult sheep. Immunopathological studies of synovial tissues from sheep at defined time points following generation of arthritis showed that there were temporal differences in the proportions of individual cell types infiltrating these tissues. The CD4:CD8 T lymphocyte ratio was significantly higher, and the the T:B and αβ:γδ T lymphocyte ratios were significantly lower in tissues from day 3 compared to day 30 following generation of arthritis. A large proportion of all three T lymphocyte subsets in SF were activated as judged by MHC class II and IL2 receptor expression. There was also temporal variation in the expression of some cell adhesion molecules by T lymphocytes in SF.

The feasibility of following inflammatory cell trafficking out of inflamed joints into the afferent lymphatics was assessed using the AIA model. Following cannulation of the popliteal pseudoafferent lymphatic duct in the hindlimb, AIA was generated in the tibiotarsal joint distal to the cannulation site. In a small number of sheep it was possible to monitor changes in the draining lymph following generation of arthritis or a flare reaction. In the acute stages of arthritis these changes were characterised by large numbers of neutrophils followed by increases in the concentration or output of lymphocytes and dendritic cells (DC). Temporal variation was observed in the expression of activation and other molecules by lymphocytes and DC in the draining lymph. Antigen uptake by different cell types in AL was demonstrated by generating arthritis using fluorescein-labelled antigen and the potential for DC to migrate from SF into the afferent lymphatics was investigated using DC labelled *in vitro* with a fluorescent dye. These results indicate that lymphatic cannulation could provide an important novel approach to studying the dynamics of the inflammatory process in joints.

CONTENTS

Abbreviations

AA	Adjuvant arthritis
AGID	Agar gel immunodiffusion
AIA	Antigen-induced arthritis
AL	Afferent lymph
ANOVA	Analysis of variance
APC	Antigen-presenting cell
Az	Azide
BSA	Bovine seurum albumin
CAEV	Caprine arthritis-encephalitis virus
CAM	Cell adhesion molecule
CFA	Complete Freund's adjuvant
CIA	Collagen-induced arthritis
CICIA	Collagen immune complex-induced arthritis
CLA	Cutaneous lymphocyte antigen
C:MC	Carpal:Metacarpal
CNS	Central nervous system
ConA	Conconavalin A
CTL	Cytotoxic T lymphocyte
DAB	Diamino-benzidine tetrachloride
DC	Dendritic cell
DTH	Delayed-type hypersensitivity
ECM	Extracellular matrix
EL	Efferent lymph
ELAM	Endothelial leucocyte-associated molecule
ELISA	Enzyme-linked immunosorbent assay
FCS	Foetal calf serum
FcR	Fc receptor
FIV	Feline immunodeficiency virus
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H+E	Haematoxylin and eosin
HEV	High endothelial venule
HIV	Human immunodeficiency virus
HSA	Human serum albumin
HSP	Heat shock protein
^{3}H-TdR	Tritiated methyl thymidine
HTLV	Human T-lymphotrophic virus
i/a	Intraarticular

IC	Immune complex
ICAM	Intercellular adhesion molecule
IFA	Incomplete Freund's adjuvant
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LAK	Lymphokine-activated killer cell
LC	Langerhan's cell
LFA	Lymphocyte function-associated antigen
LMN	Large mononuclear cell
LTB4	Leukotriene B4
LTR	Long terminal repeat
LV-IFN	Lentiviral interferon
Mab	Monoclonal antibody
MCP	Monocyte-chemoattractant protein
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MIP	Macrophage inhibitory protein
MPG	Methyl pyronin green
MVV	Maedi-Visna virus
NK	Natural killer
NSE	Non-specific esterase
ORF	Open reading frame
OVA	Ovalbumin
PAF	Platelet-activating factor
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PG	Prostaglandin
PLA	Phospholipase
PPD	Purified protein derivative
RA	Rheumatoid arthritis
RC	Radiocarpal
RF	Rheumatoid factor
SF	Synovial fluid
SI	Subintima
SIV	Simian immunodeficiency virus
SLC	Synovial lining cell

TCR	T cell receptor
TGF	Transforming growth factor
TNCC	Total nucleated cell count
TNF	Tumour necrosis factor
TT	Tibiotarsal
VAP	Vascular adhesion protein
VCAM	Vascular cell adhesion molecule
VLA	Very late antigen

CHAPTER 1

Introduction

1. Introduction

Arthritis in sheep is a common clinical entity and a major cause of carcase condemnation and hence economic loss to the sheep industry (Jones 1978). The various forms of joint disease that occur naturally in sheep can be broadly divided into two categories; disease caused by infectious agents and traumatic or degenerative joint disease. The commonest form that is recognised is caused by infectious agents (Angus 1991). However, the relationship between infectious agents and the development of chronic inflammatory joint disease in sheep is less well understood than some other species, particularly humans. Chronic synovial inflammation has been studied extensively in man, but relatively few studies have been performed on joint tissues from natural or experimental arthropathies of other species including sheep. Understanding the characteristics of these responses in sheep may provide a valuable insight into the pathogenesis of inflammatory joint disease in this species.

2. Development, structure and function of normal synovium

Synovial cavities are enclosed by a fibrous capsule that helps to maintain the apposition of the cartilage-covered ends of bones comprising the articulation. The inner lining of the joint capsule is referred to as the synovial lining or synovium. During embryonic development the synovial lining develops from the synovial mesenchyme at the periphery of the interzone between the cartilaginous precursors of synovial joints (O'Rahilly and Gardner 1978). With the invasion of blood vessels into the developing synovial lining, increasing numbers of cells appear with the histochemical characteristics of macrophages and mast cells (Andersen *et al* 1964).

The basic histological and ultrastructural characteristics of the synovial lining from normal joints of a large number of species, including sheep (Cutlip and Cheville 1973), have been described (Wylie *et al* 1964, Luckenbill and Cohen 1967, Fell *et al* 1976, Johansson and Rejno 1976, Okada *et al* 1981, Brassfield 1982, reviewed in Henderson and Edwards 1987, Edwards *et al* 1988).

Histologically, the synovial lining can be divided into two layers, the synovial lining cell (SLC) layer and the subintimal (SI) layer that merges with the joint capsule (Henderson and Pettipher 1985). The SLC layer is comprised predominantly of two different cell types with different origins and functions. These can be differentiated on the basis of electron microscopic appearance (Barland *et al* 1962), immunocytochemical (Burmester *et al* 1983) and cytochemical staining charactersitics (Wilkinson *et al* 1992). Type A cells are monocyte-derived (Edwards and Willoughby 1982) and express monocyte-macrophage antigens (Burmester *et al* 1983), whereas type B cells lack the aforementioned antigens, show high levels of uridine diphosphoglucose dehydrogenase (UDPGD) activity (Wilkinson *et al* 1992) which is an enzyme responsible for hyaluronan synthesis, and are usually described as fibroblast-like.

The SI can be defined as adipose, areolar or fibrous depending upon its predominant histological characteristics. The vascularity of the tissue varies from region to region but several authors have documented an abundance of vessels immediately beneath and amongst the SLC (Knight and Levick 1983, Wilkinson and Edwards 1989). Lymphatic vessels have also been identified in synovium from several species (Davies 1946, Wilkinson and Edwards 1991).

The functions of normal synovium have been reviewed by Henderson and Edwards (1987). These include ensuring non-adherence to, and low surface friction between other structures in the joint, providing deformable packing, controlling the volume and composition of synovial fluid (SF) and it provides most or all of the blood supply to articular cartilage.

3. Natural and experimental arthropathies in sheep

The commonest form of naturally-occuring joint disease in sheep is an inflammatory polyarthritis, which can be caused by bacterial and viral agents (Jones 1978). It is most frequently seen in groups of lambs and young adults (under 15 months of age) associated with a wide range of bacterial infections (reviewed in

Lamont 1978, Angus 1991). The most commonly isolated bacterial organisms from affected joints are *Streptococcus sps, Staphylococcus sps*, coliforms and *Erysipelothrix rhusiopathiae*. Other bacterial organisms that have been isolated less frequently include *Chlamydia psittaci* (Foggie 1977) and *Mycoplasma capricolium* (Swanepoel *et al* 1977). The usual routes of entry for these organisms are the navel, tail and castration wounds, tick bites and through the use of sheep dip contaminated with bacteria (Angus 1991) and the joints most commonly affected are the carpal, tarsal, and stifle joints (Lamont 1978). The organism most widely recognised as a cause of polyarthritis in lambs is *E. rhusiopathiae* (Radostits *et al* 1994). Both acute and chronic forms of the disease are recognised (Lamont 1979). The acute form usually affects lambs under two months of age, from which the majority of lambs recover, while the chronic form is most frequently seen in animals between two and six months of age (Jones 1978). The chronic form of the disease is characterised by joint deformities, ankylosis and erosions of the articular cartilage. Somewhat surprisingly, given the frequency of the disease, the histopathological features of synovium from naturally-infected sheep have only been reported once (Tontis *et al* 1977). The chronic synovitis in lambs up to five months post-infection was characterised by villous hyperplasia with large numbers of lymphocytes and plasma cells.

Experimentally, *Erysipelothrix* polyarthritis has been induced in lambs using living or dead organisms innoculated by a variety of routes (Marsh 1933, Piercy 1971, Lamont 1978). The clinical and pathological features of the disease generated in these ways are similar to those described for natural *E. rhusiopathiae* infection. Although chlamydial polyarthritis in lambs has been recognised in the UK, the disease has been reported to affect primarily feedlot lambs in the USA (Foggie 1977). Experimental infections with this agent closely resemble the natural disease (Cutlip and Ramsey 1973).

Arthritis affecting older adult sheep most commonly affects individual sheep rather than entire groups and its causes are less well understood. In an abbatoir

survey of sheep carcases condemned because of gross joint enlargement, Lamont (1978) reported that over 80% of the adult and broken-mouthed sheep (aged over 4 years) had uni- or bilateral osteoarthritis of the elbow joints. Under 5% of these sheep were condemned because of carpal or tarsal joint enlargement and the characteristics of the disease affecting these joints were not described.

The role of bacterial organisms in the pathogenesis of arthritis in older sheep is very poorly understood. Bacterial organisms were rarely isolated from SF from the joints of older sheep in the study by Lamont (1978) but no attempt was made to culture organisms from the synovial lining which would have increased the likelihood of isolating bacteria if they were present (Goldenberg 1993). Although *E. rhusiopathiae* has been implicated in the pathogenesis of chronic inflammatory arthritis in older sheep, the organism is rarely isolated from chronically arthritic joints (Lamont 1979, Radostits *et al* 1994). Evidence from experimental infections in pigs, rabbits and sheep have demonstrated the capacity of non-viable fragments of the bacteria to cause a polyarthritis (Freeman 1964, Piercy 1971, White and Puls 1971) and although the reasons for chronicity are poorly understood, variation in the virulence of different strains (Lamont 1979), long-term persistence of bacterial antigen in synovium (Drommer 1982) and the development of autoimmune reactivities (Timoney 1971) have been evoked as mechanisms perpetuating chronic synovial inflammation.

Although bacteria are the commonest cause of inflammatory joint disease in sheep, Maedi-Visna virus (MVV) has also been associated with the development of chronic inflammatory arthritis in sheep in the USA (Cutlip *et al* 1985a, Cutlip *et al* 1986, Kennedy-Stoskopf *et al* 1989) and the UK (Harkiss *et al* 1991, Watt *et al* 1992a). Although all breeds would appear to be equally susceptible to infection with the virus, there is evidence to suggest that some breeds or strains of sheep are more susceptible to develop disease than others (Cutlip *et al* 1986). Arthritis associated with natural MVV infection has been reported to affect primarily sheep of the Border Leicester and Texel breed (Cutlip *et al* 1986, Harkiss *et al* 1991).

Sheep have rarely been used as a host species for the investigation of inflammatory joint disease other than that caused by infectious agents. However, a collagen immune complex-induced arthritis (CICIA) has been generated in adult sheep as part of a study to investigate the lymphatic drainage of inflamed joints (Thorpe *et al* 1992)(discussed in section 12).

With the discovery that Caprine arthritis-encephalitis virus (CAEV) was a cause of chronic arthritis in goats (Crawford *et al* 1980) and that sheep infected with MVV could also develop similar arthritic lesions, most recent research into the pathogenesis of chronic inflammatory joint disease in sheep and goats has investigated the role that these agents play in this disease process. These studies have relevance to a broader understanding of this disease process in other species, including humans. Viruses have been implicated in the pathogenesis of Rheumatoid arthritis (RA) in humans (Schumacher *et al* 1975, Ford 1993, Stransky *et al* 1993) although, to date, there is no direct evidence linking viral infection and the development of this disease. Additionally, with the emergence of Human Immunodeficiency virus (HIV) as a major cause of disease in humans and the increasing number of reports of various forms of inflammatory arthritis associated with infection with this virus (Buskila and Gladman 1990), there has been increasing interest in the potential role of retroviruses in the pathogenesis of a range of rheumatic disorders, many of which are considered to be autoimmune (Kalden *et al* 1991, Kalden and Gay 1994).

4. The role of retroviruses in the pathogenesis of arthritis

4.1. Classification, morphology and replication of retroviruses

The family Retroviridae is divided into 3 subfamilies, Oncovirinae, Lentivirinae and Spumavirinae (Coffin 1990). The retroviruses that have been reported to be associated with arthritis belong to the first 2 subfamilies. Of the Oncovirinae, only some of the Mammalian C-type viruses and Human T-cell lymphotropic virus

(HTLV-1) have been associated with the development of rheumatic disorders. Of the mammalian C-type viruses, in humans, large numbers of defective, non-replicating endogenous proviruses have been found throughout the genome (Coffin 1990). Members of the Lentivirinae that have been reported to be associated with the development of inflammatory arthritis in their respective host species include MVV, CAEV, Simian Immunodeficiency virus (SIV) and HIV. These viruses are quite similar in virion structure, genome organisation and mode of replication. Although most research has been conducted on HIV, MVV was the first lentivirus to be described (Sigurdsson 1954). The genome of MVV is diploid and consists of 2 lengths of single-stranded RNA which encode 3 basic structural genes, *gag, pol* and *env* (Sonigo *et al* 1985). These encode proteins for the internal nucleocapsid, or core proteins, a reverse transcriptase which converts the single-stranded RNA to double-stranded DNA, and the envelope glycoproteins gp135 and gp41, respectively. These regions are flanked by long terminal repeats (LTR) that contain promotor and enhancer elements. Additionally, there are open reading frames (ORF) that encode tat, rev and vif which act as regulatory proteins (Toohey and Haase 1994).

Virus entry into a cell is initiated by attachment to its cell surface receptor. The receptors for MVV and CAEV are not known, although for MVV it has been demonstrated that MHC class II may act as a component (Dalziel *et al* 1991) and antibodies to a 50kD protein block infection of sheep choroid plexus and goat synovial membrane cells *in vitro* (Crane *et al* 1991). In a similar fashion to HIV, antibody-mediated enhancement of infection has been shown for MVV (Jolly *et al* 1989). Following receptor engagement, virus envelope and host cell membranes fuse and the nucleocapsid enters the cytoplasm of the cell. The reverse transcriptase enzyme mediates the synthesis of double-stranded DNA from the viral genomic RNA, primarily in the nucleus of the cell (Haase *et al* 1982). The vast majority of viral DNA thus manufactured remains as non-integrated linear proviral DNA in the nucleus (Haase *et al* 1984). For expression of this provirus to occur,

the viral LTR provides signals recognised by the cellular transcription machinery. When productive infection occurs, viral RNA rapidly accumulates in the cell, a process that is enhanced by tat (Gabzuda *et al* 1989). Highly productive infection is a feature of viral replication in certain permissible cell lines *in vitro*. In this situation high titres of the virus are produced and cell fusion occurs (Thormar 1963). However, *in vivo*, viral replication is highly restricted with the block on virus growth occuring at the level of viral RNA transcription (Haase *et al* 1977, Brahic *et al* 1981). Following experimental infection with MVV, up to 2% of cells in infected tissues [alveolar macrophages in the lung, peripheral blood monocytes and choroid plexus cells in the central nervous system (CNS)] express viral RNA with an average copy number of 50 to 150 per cell (average of 5000 per cell in tissue culture). Of these RNA positive cells it has been estimated that only 0.001% express viral protein (reviewed in Haase 1986). Similar studies of tissues from naturally-infected animals have also shown that the numbers of cells expressing viral RNA or viral protein are low (Zink *et al* 1990, Brodie *et al* 1995). The reasons for this restriction in viral growth *in vivo* are unclear.

Cells of the monocyte-macrophage lineage are believed to be the only cell types capable of supporting productive infection *in vivo* (Gendelman *et al* 1985). However, on the basis of *in situ* hybridisation and immunocytochemical studies to detect the presence of viral mRNA and protein respectively, it has been shown that a variety of other cell types including epithelia and blood vessel endothelium may be infected *in vivo* (Georggson *et al* 1989, Zink *et al* 1990, Brodie *et al* 1995). The implications of infection of these other cell types is not known. It has also been suggested that dendritic cell (DC) precursors may be the major resevoir of infected cells in peripheral blood but the criteria used for their identification in this study did not exclude the possibility that other cell types, such as monocytes, were the source of the virus (Gorrell *et al* 1992).

4.2. Immune responses to MVV and CAEV

The immune responses to viral antigens are thought to play a very important role in the development of lesions in the target organs of infected animals. This has been highlighted by the demonstration that immunosuppression prevents the development of lesions in the central nervous system (CNS) in sheep experimentally-infected with MVV (Nathanson *et al* 1976).

4.2.1. Humoral immunity

Within several weeks of infection precipitating and complement-fixing antibodies are detectable in the sera of infected animals (Sihvonen *et al* 1981). Neutralising antibodies usually appear slightly later (several months) during natural infections (Sihvonen *et al* 1981). Antibodies of the IgG1 subclass directed to the envelope glycoproteins form the major component of the antibody response although lower levels of antibody are also produced against the core proteins (Griffin *et al* 1978, Sihvonen *et al* 1981, Johnson *et al* 1983a, b). No antibody-dependent cell-mediated cytotoxicity (ADCC) has been demonstrated in the sera from infected animals probably because in sheep this is mediated by IgG2 antibodies which have not been detected in infected sheep or goats (Johnson *et al* 1983b, Reyburn *et al* 1992). The role of these antibodies in preventing dissemination of the virus throughout the body and in disease pathogenesis are unclear. Antigenic variants appear during the course of infection as a result of antigenic drift of the env proteins. These are not recognised by the antibodies generated to the original viral isolate and it has been suggested that clonal expansion of neutralisation-resistant variants in the joints of CAEV-infected goats is the basis of recurrent antigenic stimulation that contributes to disease progression (Cheevers *et al* 1991).

4.2.2. Cell-mediated immunity

Proliferative T lymphocyte responses to viral antigens are detectable in the peripheral blood of chronically infected sheep and goats. Although some authors have found these to be transient or irregular (Griffen *et al* 1978, Larsen *et al* 1982), others have shown them to be continuously present (Reyburn *et al* 1992). The latter report showed that this response was mainly due to CD4+ T cells but some animals were found to have CD8+ T cell proliferative responses. The characteristics of the cell-mediated reponses in target tissues are less well understood. *In vitro* proliferative responses to viral antigens have been shown for lymphocytes from synovial fluid (SF) from a small number of CAEV-infected goats (Banks *et al* 1987) and in the efferent lymph (EL) of sheep experimentally-infected with MVV (Bird *et al* 1993).

CD8+ cytotoxic T cell precursors have been detected in the peripheral blood of sheep and goats infected with these viruses (Lichtensteiger *et al* 1993, Blacklaws *et al* 1994, Lee *et al* 1994). These responses were shown to be MHC- and virus-variant restricted. The appearance of neutralisation variants not recognised by CTL precursors may provide another means whereby the virus can escape recognition by the immune system in a similar fashion to that shown for HIV in man (Phillips *et al* 1991). The role of other anti-viral effector cells such as natural killer (NK) cells and lymphokine-activated killer (LAK) cells (Whitton and Oldstone 1990) is not known although it has been suggested that LAK cells may contribute to the cytotoxic responses observed *in vitro* (Lee *et al* 1994a).

4.3. Evidence of immune dysfunction in sheep and goats infected with lentiviruses

Unlike HIV in man, there is very little evidence that lentiviral infection of sheep or goats results in a state of immunosuppression. One report of a small number of arthritic MVV-infected sheep did demonstrate depression of the CD4:CD8 T lymphocyte ratios in peripheral blood (Kennedy-Stoskopf *et al* 1989)

but this has not been shown in other studies (Bird *et al* 1994). However, there is some evidence that infected sheep may show mild forms of immune dysfunction. MVV-infected sheep have been shown to have decreased delayed-type hypersensitivity (DTH) responses to mycobacterial purified protein derivative (PPD)(Dr. N. Watt, personal communication) and primary antibody responses to ovalbumin (Blacklaws *et al* 1994a). Infections with *Pasteurella multocida*, an uncommon pathogen in sheep, have been reported to be more frequent in the lungs of MVV-infected sheep (Watt *et al* 1992a). Although *in vitro* peripheral blood T lymphocyte responses to protein antigens and mitogens are reported to be normal in MVV-infected sheep (Blacklaws *et al* 1994), for CAEV-infected goats, exaggerated responses to mitogens are sometimes present, although the significance of this is unclear (Banks *et al* 1989).

4.4. Pathogenesis of ruminant lentiviral arthritis

MVV and CAEV are transmitted from infected dams to their newborn offspring via colostrum and milk. Transmission of the virus in older animals occurs by direct contact which primarily involves oral ingestion of secretions from infected animals (Kennedy-Stoskopf *et al* 1987). Clinical signs of joint disease are not usually seen before animals are at least 2 years of age (Kennedy-Stoskopf *et al* 1989). The onset of inflammatory joint disease is usually slow and insidious, although in some cases it may be acute (Crawford and Adams 1981, Kennedy-Stoskopf *et al* 1989). Although the primary target organs in MVV (lungs, CNS and to a lesser extent the mammary gland and joints) and CAEV (joints in adults and the CNS in kids) infection are different, subclinical inflammatory changes are frequently present in the target tissues in both infections (Crawford *et al* 1980, Haase 1986). Experimentally, the induction of arthritis with these viruses has proved to be easier in newborn animals than in adults (Cheevers *et al* 1988, Dr G. Harkiss, personal communication). Although there are a number of possible

explanations for this phenomenon, there may be characteristics of joint tissues in very young animals that predispose them to the development of disease.

The pathological changes in joint tissues from arthritic sheep and goats are similar and are characterised by chronic inflammatory and degenerative changes that can result in severe erosive disease in a small proportion of animals (Banks *et al* 1989). The reasons for the differences in clinical expression of disease between the two infections are not clear. The two viruses are closely related morphologically and immunologically (Dahlberg *et al* 1981, Pyper *et al* 1984, Cheevers *et al* 1984) but differences do exist in genomic nucleic acid sequences, particularly in the *env* gene (Pyper *et al* 1984). Interestingly, each species can be cross-infected with virus from the other species and develop clinical signs of arthritis (Banks *et al* 1983).

Infected macrophage precursors in bone marrow provide a reservoir of latently infected cells (Gendelman *et al* 1985). Because many of the cells in synovial tissues have monocyte precursors (section 2), this provides a mechanism whereby latently infected cells can gain access to joints that possess a synovial lining, tendon sheaths and bursae. This mechanism of virus entry into tissues has been referred to as a Trojan horse mechanism for the spread of virus (Haase 1986). Activation and maturation of infected monocytes is then necessary for productive infection to occur (Narayan *et al* 1983), although the precise activation requirements are not fully understood. Studies of mice transgenic for the LTR of MVV have shown that *in vitro* activation of peritoneal macrophages via a protein kinase C pathway was necessary for increased LTR expression (Small *et al* 1989). Although it is known for HIV that a number of cytokines [granulocyte-macrophage colony stimulating factor (GM-CSF), tumour necrosis factor (TNF)α and β, interleukins (IL)1β and 6] can upregulate virus replication *in vitro* (Rosenberg and Fauci 1990, Tornatore *et al* 1991), their role in the regulation of virus replication in ruminants is not known.

The mechanisms responsible for the development of chronic synovial inflammation are unclear. The importance of immune-mediated responses has been demonstrated by the ability to suppress the development of inflammatory lesions in the CNS of sheep experimentally-infected with MVV using cyclophosphamide and antisera to sheep thymocytes (Nathanson *et al* 1976). Additionally, induction of an antigen-induced arthritis (AIA) in CAEV-infected goats resulted in a more severe disease compared to non-infected individuals in the absence of evidence of increased viral replication or local anti-viral specific immune responses (Banks *et al* 1987). This suggests that factors other than anti-viral immune responses in the joints of these animals may contribute to the progression of arthritis.

It has been shown for CAEV-infected goats that the severity of arthritis correlates with local production of antibodies to the envelope glycoproteins (Johnson *et al* 1983a,b, Knowles *et al* 1990) and that the appearance of clinical joint disease is associated with the presence of antibodies to the immunodominant epitopes of the gp38 transmembrane protein in serum (Bertoni *et al* 1994). The role of these antibodies in the pathogenesis of MVV-related disease is less fully understood. Although immune complexes (IC) of the viral core protein p25 and its antibody have been demonstrated in the EL of sheep experimentally-infected with MVV (Bird *et al* 1993) and in the articular tissues of some arthritic sheep (Harkiss *et al* 1995a), IC were not found in the SF of arthritic CAEV-infected goats by C1q-binding assay (Zwahlen *et al* 1987).

Very little is known of the role of cytokines in the pathogenesis of lentiviral arthritis in ruminants. Following interaction with virally-infected macrophages, T lymphocytes produce an interferon [usually referred to as lentiviral-interferon (LV-IFN)] which has properties of both α- and γ-IFN, in addition to its own unique properties (Narayan *et al* 1985, Kennedy *et al* 1985). *In vitro*, this cytokine inhibits the maturation and proliferation of monocytes, upregulates MHC class II expression and directly and indirectly inhibits viral replication (Kennedy *et al* 1985, Zink and Narayan 1989). Low levels of LV-IFN-like activity have been

demonstrated in the SF from arthritic MVV-infected sheep (Kennedy-Stoskopf *et al* 1989) and elevated MHC class II expression was found to be increased on SF macrophages from the joints of MVV-infected sheep compared to non-infected control animals (Harkiss *et al* 1991). It has also been reported that following activation *in vitro,* lymphocytes from CAEV-infected goats release a factor that is mitogenic for macrophages (Jutila and Banks 1988). Although this mitogen has not been characterised, the observation raises the possibility that any factor responsible for lymphocyte activation locally in joints could lead to macrophage responses that contribute to the development of chronic synovitis.

High levels of expression of IL-1β and TNFα have been detected by *in situ* hybridisation in the synovial lining from arthritic CAEV-infected goats together with low or absent levels of the T lymphocyte-derived cytokines IL-2 and γ-IFN (Lechner *et al* 1994). Also, IL-8 was not detected by this method which may provide one explanation for the paucity of neutrophils in SF of these animals (Lechner *et al* 1994). Similar studies have not been performed on other inflammatory arthropathies of sheep or goats for comparison but the predominance of macrophage-derived cytokines is perhaps expected given their abundance in the joints of humans with inflammatory arthropathies (Arend and Dyer 1990).

Other immunopathogenic mechanisms that may contribute to the pathogenesis of chronic synovitis include immune responses to heat shock proteins (HSP) in synovium (Harkiss *et al* 1995b) and to host tissue components (Harkiss *et al* 1993). Although elevated levels of rheumatoid factors (RF) and other autoantibodies have been detected in serum of infected sheep (Harkiss *et al* 1993), these have not been detected in the serum of arthritic goats (Zwahlen *et al* 1985, Kennedy-Stoskopf *et al* 1987).

The observation that chondrocytes in cartilage from a small number of sheep not infected with MVV expressed antigens recognised by polyclonal antibodies to recombinant MVV viral core protein p25 and synthetic analogues of MVV tat and vif proteins, suggests that molecular mimicry and cross-reactivity may also play a

role in the pathogenesis of arthritis in these sheep, although there is no evidence from functional studies to support this (Harkiss *et al* 1995a). Similar observations have been made from normal human tissues that were found to express antigens recognised by monoclonal antibodies (Mab) to the regulatory proteins of HIV (Parmentier *et al* 1992) and the p19 core protein of HTLV-1(Palker *et al* 1985). Additionally, SLC and cells within blood vessel walls in synovium from a proportion of humans with RA have been shown to express antigens recognised by Mab to some of the core proteins of HTLV-1 (Ziegler *et al* 1989).

4.5. Retroviral arthritis in other species

A wide range of arthritic disorders associated with HIV infection have now been recognised in humans, including arthralgias, reactive and psoriatic arthritis and a seronegative arthropathy referred to as HIV-associated arthropathy (reviewed in Espinoza *et al* 1989, Buskila and Gladman 1990). Most of these diseases are also found in humans not infected with HIV, but some have been reported to be present at a higher frequency in infected individuals (Berman *et al* 1988, Forster *et al* 1988). The pathogenesis of inflammatory arthritis in these individuals is unclear and complicated by the wide range range of cell types capable of being infected with the virus and the frequent opportunistic infections that these patients suffer (Levy 1993). HIV has been isolated from DC and lymphocytes from SF of affected individuals (Withrington *et al* 1987, Hughes *et al* 1990) but the role of the virus in the initiation or perpetuation of synovial inflammation is not known.

Some humans infected with HTLV-1 have been reported to develop a chronic inflammatory polyarthritis (Kitajima *et al* 1989, Sato *et al* 1991). Subsequently it has been shown that mice transgenic for HTLV-1 develop an erosive inflammatory polyarthritis (Iwakura *et al* 1991, Yamamoto *et al* 1993) and human synovial cells infected or transfected with the virus proliferate and produce granulocyte-macrophage colony-stimulating factor (GM-CSF) (Nakajima *et al* 1993, Sakai *et al* 1993), a cytokine considered to be of importance in upregulating the expression of

MHC class II in synovial tissues (Xu *et al* 1989). Although it has been shown that some antigens expressed in the synovium from humans with RA are identified by antibodies to the core proteins of HTLV-1 (Ziegler *et al* 1989), investigations utilising the polymerase chain reaction (PCR) using primers to conserved regions of the *pol* gene from a number of exogenous lentiviruses failed to demonstrate the presence of these viruses in tissues from humans with RA (DiGiovine *et al* 1994). However, an A-type retrovirus has been isolated from humans with Sjogrens syndrome (Flescher *et al* 1992) and antibodies to retroviral proteins have been detected in the serum of patients with this disorder (Talal *et al* 1990) suggesting that these viruses may play a role in some rheumatic disorders of humans.

Up to 45% of Macaques experimentally-infected with SIV have been reported to develop an inflammatory polyarthritis (Roberts and Martin 1991) and this disease has also been observed in cats infected with Feline immunodeficiency virus (FIV)(Dr. D. Bennett, University of Liverpool, personal communication). Inflammatory joint disease associated with viruses of the lentivirinae infecting other species have not been reported.

Numerous animal models of chronic inflammatory arthritis have been described in the literature (reviewed in Sokoloff 1984, Crofford and Wilder 1994). Most have been generated in the hope that they will elucidate pathogenetic mechanisms that may operate in natural arthritic diseases of humans, particularly RA. Although no animal model of arthritis shares identical clinical and pathological features to this condition of humans, they do display some similarities.

It has been suggested that the pathogenesis of lentiviral arthritis in ruminants may bear some similarity with the chronic inflammatory process generated during AIA (Harkiss 1994). Both disease states are characterised by cell-mediated and humoral immune responses to the antigen, significant local antigen-specific B cell responses and persistence of antigen in articular tissues. The histological features of synovium are similar and both diseases can result in the erosion of articular

cartilage. Clearly, there are also likely to be major differences in the pathogenesis of these two diseases. Viral antigens in the joints of sheep could be processed and presented by antigen-presenting cells (APC) to T lymphocytes associated with MHC class I or class II whereas during AIA, most antigen is likely to be presented to these cells associated with MHC class II, although it is possible for exogenous antigens to enter MHC class I processing pathways (Germain 1994). Of particular relevance in this respect is the recent demonstration that cytolytic T cell responses were generated to ovalbumin (OVA) following immunisation of mice to this antigen using complete Freund's adjuvant (CFA)(Ke *et al* 1995).

Studies of different inflammatory arthropathies in humans has shown that they frequently share common pathological and immunopathological features despite putative differences in aetiology (Cooper *et al* 1981, Lindblad and Hedfors 1985). For ruminants there is a paucity of information regarding the pathogenesis of inflammatory arthritis, regardless of its aetiology. Hence, comparison of different disease states with different aetiologies may provide an insight into the characteristics of synovial immune responses in this species.

5. Antigen-induced arthritis: An overview

AIA was first described by Dumonde and Glynn (1962) who generated the disease in rabbits by immunising them to autologous or heterologous fibrin followed by intraarticular injection of the same antigen. Since its first description, numerous antigens have been employed to induce the disease (reviewed in Cooke 1988) and, in addition to rabbits, it has been generated in mice (Brackertz *et al* 1977a), rats (Dijkstra *et al* 1987), guinea pigs (Dreher 1982) and goats (Banks *et al* 1987).

Immunisation to an antigen using CFA results in the development of cell-mediated and humoral immunity to that antigen. Antigen injected intraarticularly rapidly diffuses into the articular tissues where it complexes with antibody (Cooke and Jasin 1972). These immune complexes activate complement by the classical

pathway resulting in an influx of neutrophils. Recent studies of mice deficient in the γ-subunit of the Fc receptor (FcR) have suggested a central role for these receptors in initiating the immune complex inflammatory response in the skin (Sylvestre and Ravetch 1994). The cell type in the skin implicated in mediating this response is the mast cell. Whether the same is true for the development of Arthus reactions in joints is not known, although mast cell-deficient mice do not show attenuated responses during the induction of AIA (Van den Broek *et al* 1987). The importance of the acute inflammatory response for the subsequent development of chronic synovitis has been demonstrated by complement and neutrophil depletion studies (Rawson and Torralba 1967, Hollister *et al* 1973, Van Lent *et al* 1992), and more recently by the *in vivo* use of Mab to block neutrophil entry into joints (Jasin *et al* 1992).

Following the rapid influx of neutrophils, T and B lymphocytes, macrophages and dendritic cells migrate into the synovial lining (Dijkstra *et al* 1987, Verschure *et al* 1989, Wilkinson *et al* 1993a). This chronic synovitis persists for extended periods of time and frequently accompanies erosive changes to the articular cartilage (Dumonde and Glynn 1962). Antigen persists as IC in the superficial regions of the dense collagenous hypo- and avascular tissues in the joint, particularly the ligaments, menisci and articular cartilage (Cooke and Jasin 1972). Despite the persistence and severity of the chronic synovial inflammation, the half-life of antigen in synovium has been estimated to be substantially less than that for the dense collagenous structures in the joint (Hollister and Mannik 1974). Studies in mice using radiolabelled antigen have failed to detect antigen by autoradiography in synovium 4 days after intraarticular injection (Van den Berg *et al* 1982) and similar studies in the rabbit have shown that less than 1% of the locally-retained antigen at 6 weeks following induction of the disease is located in this tissue (Cooke and Jasin 1972). Antigen retention in the joint is influenced by the size and density of the cation groups on the outer surface of the antigen (Van Lent *et al* 1987) and its electrical charge (Van den Berg *et al* 1984), and has been detected in

the joints of rabbits up to 132 days following generation of the arthritis (Consden *et al* 1971).

The importance of cell-mediated immunity to the antigen for the generation and persistence of a chronic synovitis has been demonstrated by the ameliorating effect of anti-lymphocyte serum (Goldberg *et al* 1974), cell transfer experiments (Brackertz *et al* 1977b) and the demonstration that immunisation using Freund's incomplete adjuvant (FIA) results in a transient synovitis only (Fox and Glynn 1977, Henderson *et al* 1982, Pettipher and Henderson 1988). Antigen-specific antibody has been shown to account for up to 40% of the local antibody production at 6 weeks following induction of arthritis (Cooke *et al* 1972), suggesting that B lymphocytes with a specificity for antigens other than that used to induce the arthritis are also stimulated to differentiate, although the specificities of these antibodies has not been determined.

The possibility that autoimmune reactivities may develop to host tissue components during the course of the AIA was first suggested by Glynn (1968) and evidence to support this has been provided by Champion *et al* (1981, 1983) who demonstrated splenic T lymphocyte proliferative responses to cartilage proteoglycan and collagen types I, II and III in a small number of rabbits following induction of AIA. However, these responses were not observed in all rabbits, were only observed in the first few months following induction of arthritis and the stimulation indices in the majority of rabbits were low. Additionally, low levels of antibodies to proteoglycan but not collagens were detected in serum from a proportion of affected rabbits. More recently the appearance of similar reactivities following the generation of AIA in mice and rats has been documented (Brauer *et al* 1993a,b). The role that these responses may play in the perpetuation of chronic synovitis is unclear.

Since its original description AIA has been used to study a variety of pathological processes that occur in joints during the acute and chronic phases of the inflammatory response, some of which may potentially be relevant to the

understanding of lentiviral arthritis. Proteoglycans are an important component of articular cartilage and their depletion is a feature of AIA (Pettipher *et al* 1989, Henderson *et al* 1990) and has been observed in cartilage from sheep with lentiviral arthritis (Harkiss *et al* 1995a). Studies involving neutropaenic rabbits (Pettipher *et al* 1988) and mice whose neutrophils are deficient in elastase and cathepsin G (Pettipher *et al* 1990) have shown that these cells are not responsible for the acute loss of proteoglycan from articular cartilage during the early stages of AIA . However, intraarticular injection of IL-1 in these animals does result in an acute loss of proteoglycan from the articular cartilage (Pettipher *et al* 1988). Although these results suggest that the loss of cartilage proteoglycan is not mediated by infiltrating leucocytes, it is still unclear whether it results from direct enzymatic attack from activated resident synovial cells (Henderson and Glynn 1981, Hembry *et al* 1993), activation of chondrocytes by IL-1 or other cytokines, or as a consequence of autolysis following chondrocyte death which occurs during AIA, particularly when high doses of antigen are used to generate the arthritis (Howson *et al* 1984).

Other features of chronic inflammatory arthritis that have been investigated using AIA as a model system include the production and role of cytokines and metalloproteinases (Henderson *et al* 1988, Henderson *et al* 1990, Zarco *et al* 1992, Hembry *et al* 1993) and the proliferation and metabolism of SLC (Henderson and Glynn 1981, Henderson *et al* 1982, Etherington *et al* 1988) and their role in the regulation of synovial inflammatory responses (Van Lent *et al* 1993). It has also been used as a model system with which to examine the effects of treatment with Mab to cell adhesion molecules (CAM)(Jasin *et al* 1992) and more recently to determine the effects of induction of peripheral tolerance on the course of the arthritis (Jacobs *et al* 1994a)

5.1. Flare reactions following the generation of AIA.

Flare reactions are acute exacerbations of inflammation that occur during chronic synovial inflammation and are a common clinical manifestation of chronic inflammatory joint disease in many species. Although initially generated experimentally in the rabbit (Cooke and Maeda 1980), the phenomenon has been most extensively studied in the mouse (Van de Putte *et al* 1983). Although this response was initially thought to be specific for the antigen that had originally induced the inflammatory response (Lens *et al* 1984a), they can also be triggered by the intraarticular administration of IL-1 (Van de Loo *et al* 1992). Pretreatment with anti-lymphocyte serum (Lens *et al* 1984b) and with Mab to MHC class II (Van den Broek *et al* 1986) suppress the reaction although decomplementation did not affect the response (Lens *et al* 1984b). More recently it has been shown that *in vivo* Mab treatment to CD4+ T lymphocytes abrogates the reaction (Jacobs *et al* 1994b) which together with the previous observations suggests that presentation of antigen associated with MHC class II to CD4+ T lymphocytes may be an important feature of these responses.

6. The involvement of different cell populations in the pathogenesis of chronic synovial inflammation

Cellular immunological mechanisms are now considered to be central to the pathogenesis of the chronic synovial inflammation. The phenotype and function of the cells involved in this process are reviewed below.

6.1. Lymphocytes

Chronic synovial inflammation in virtually all natural and experimental arthropathies is characterised by the the presence of lymphoid cells. Most of the phenotypic and functional characterisation of lymphocytes in chronically inflamed joints has been performed on cells from the affected joints of humans with RA or reactive arthropathies. A large number of studies have characterised the T and B

lymphocytic infiltrate of synovial tissues from humans (reviewed in Henderson and Edwards 1987) but fewer studies have been performed on synovial tissues from natural or experimental animal models of arthritis. Of the T lymphocyte subsets, most studies of synovium from humans have shown that CD4+ T cells generally predominate over the other T lymphocyte subsets regardless of the clinical categorisation of the disease (Lindblad and Hedfors 1985). The same is also true for synovium from a number of animal models of arthritis including collagen-induced arthritis (Klareskog *et al* 1983), Staphylococcal arthritis in rats (Bremell *et al* 1994), Streptococcal cell wall-induced arthritis (Allen *et al* 1985), CICIA in sheep (Thorpe *et al* 1992) and AIA in rats (Verschure *et al* 1989).

Although the majority of T lymphocytes in all tissues investigated express the αβ T cell receptor (CD4+ and CD8+ T lymphocytes), T lymphocytes expressing the γδ TCR have been identified in synovial tissues of humans (Holoshitz *et al* 1989) although they have rarely been identified in the arthritic joints from other species (Harkiss *et al* 1990). γδ T cells are found at a low frequency in the peripheral blood of humans and rodents but in young ruminants they can comprise up to 50% of the T lymphocytes in peripheral blood (Hein and MacKay 1991). The restriction element for these cells is not as clearly defined as for αβ T cells but clones recognising antigen associated with MHC classes I, II and Ib have been identified (Haas 1993). In sheep, their restriction element is unknown. In addition to the three major subsets of T lymphocytes, NK cells and LAK cells have been identified in the joints of human patients with RA (Hendrich *et al* 1991, Yamaga *et al* 1993), although their role in the pathogenesis of this disease and in arthropathies in other species is not clear. B lymphocytes are usually present in smaller numbers than T lymphocytes in synovial tissues from humans with inflammatory arthropathies (reviewed in Henderson and Edwards 1987) and in synovium from those laboratory animal models of arthritis that have been investigated using immunocytochemistry to characterise the inflammatory infiltrate (Allen *et al* 1985, Verschure *et al* 1989, Bremell *et al* 1994).

A very large number of studies have been performed to characterise the activation status of T lymphocytes in SF of humans with inflammatory arthropathies. These have shown that a large proportion of the SF T lymphocytes are activated, as judged by the elevated expression of MHC class II (Burmester *et al* 1981) and many of the CAM (Takahashi *et al* 1992), compared to T lymphocytes in peripheral blood. However, only a small proportion of SF T lymphocytes express other molecules indicative of activation such as receptors for interleukin 2 (IL2r) or transferrin (Burmester *et al* 1984). This discordant expression of MHC class II and IL2r has been referred to as a state of "frustrated activation" (Pitzalis *et al* 1987). The mechanisms whereby T lymphocytes are activated at sites of chronic synovial inflammation are unclear but recent evidence suggests that cytokines and interactions with components of the extracellular matrix (ECM), in the absence of TCR engagement, may play an important role (Linardopoulos *et al* 1992, Unutmaz *et al* 1994, Sturmhofel *et al* 1995). Very few studies have investigated the activation status of synovial T lymphocytes in animal models of arthritis, one explanation for which is the paucity of synovial tissues in most of the species used for these investigations.

SF T lymphocytes from arthropathies in humans are almost exclusively of a memory phenotype, that is they express the low molecular weight isoform of CD45, designated CD45RO (Pitzalis *et al* 1988a) and elevated levels of most CAM (Takahashi *et al* 1992). However, precisely how the expression of the above molecules relates to memory status is contentious (Gray 1993). Recent studies in the rat suggest that the various isoforms of CD45 distinguish between those that have encountered antigen in the recent past, that is they reflect activational status rather than a permanent state of differentiation, and therefore may be unsuitable as markers for true memory status (Bell and Sparshot 1990, Sparshot and Bell 1994). T lymphocytes with a memory phenotype are not only found in inflamed joints but are the predominant T lymphocyte population at other sites of chronic inflammation that have been investigated in humans and sheep (Pitzalis *et al* 1988a,

MacKay *et al* 1992a). Recall responses are confined to T lymphocytes with a memory phenotype and their activation requirements are less stringent compared to naive T cells, ensuring that more rapid responses are made should antigen be encountered (MacKay 1991).

Considerable controversy surrounds the role that lymphocytes play in the pathogenesis of chronic synovitis (Firestein and Zvaifler 1990, Kingsley and Panayi 1994). This controversy has arisen for a number of reasons. The number of T lymphocytes that are specific for the inciting antigen at sites of chronic inflammation is known to be very low (McCluskey *et al* 1963). Despite evidence of activation, very small numbers of T cells in joints are in the process of division indicated by the low levels of DNA synthesis (Bonvoisin *et al* 1984), Ki67 expression (Lalor *et al* 1987) and tritiated thymidine incorporation (Bergroth *et al* 1985).

Virtually all functional assays of joint-derived T lymphocytes have been performed with cells from the joints of humans with inflammatory arthropathies. For SF T lymphocytes from humans with inflammatory arthropathies, proliferative responses have been obtained *in vitro* to bacterial organisms (Viner *et al* 1991), human and mycobacterial heat shock proteins (HSP)(Pope *et al* 1992) and other self antigens (Van Schooten *et al* 1994). Also, cytotoxic T lymphocyte responses have been obtained *in vitro* to host tissue components (Gau *et al* 1994), human and mycobacterial HSP (Li et al 1992).

Despite this controversy regarding the importance of T lymphocytes in the pathogenesis of chronic inflammatory arthritis in humans there is considerable evidence from animal models of arthritis that T lymphocytes can play an important role in its development. Foremost amongst these was the discovery that some T cell clones from rats with adjuvant arthritis can transfer the disease to unaffected recipients whilst other clones can transfer resistance to develop the disease (Holoshitz *et al* 1983). Similarly, T lymphocytes from affected animals can transfer streptococcal cell wall-induced arthritis (Dejoy *et al* 1989) and collagen-induced

arthritis (CIA)(Holmdahl *et al* 1986) to naive recipients and AIA can be generated in mice by transfer of T lymphocytes from an immunised animal to a naive host if antigen is administered intraarticularly at the time of cell transfer (Brackertz *et al* 1977b).

In comparison, with the exception of immunogobulin expression, less is known regarding the phenotype and activation status of B lymphocytes at sites of chronic synovial inflammation. Clearly some B lymphocytes differentiate into plasma cells and produce antibody but the specificity of the antibody produced by large numbers of these cells is frequently not known (Egeland *et al* 1982), even in an experimental situation where the antigen inducing the immune response is known (Cooke and Jasin 1972). It is also unclear whether most plasma cells have differentiated locally, following activation by antigen or by a polyclonal stimulus, or result from the synovium being infiltrated by plasmablasts generated in the lymph nodes draining the joints. Recent evidence from humans with RA suggest that some RF B cell precursors are found with a higher frequency in SF than in peripheral blood suggesting preferential homing, local expansion or retention of these cells within the joint tissues (Moynier *et al* 1992).

6.2. Cells of the monocyte-macrophage lineage

Large numbers of monocyte-derived macrophages accumulate in chronically inflamed synovia. Although macrophages are derived from the same precursor in the peripheral blood (Van Furth 1986), they display considerable phenotypic heterogeneity in synovium (reviewed in Highton and Palmer 1994) and functionally distinct subsets have been recognised in synovial tissues from human patients with RA (Koch *et al* 1991).

Although most of the phenotypic and functional characteristation of macrophages has been performed on cells from the joints of humans with RA, there is considerable evidence from animal models of arthritis that these cells may play a pivotal role in the pathogenesis of the disease. During the development of AA in

rats, prior to the appearance of inflammatory changes in the synovium, macrophages within the synovial lining cell layer show signs of activation (Johnson *et al* 1986, Lopez-Bote *et al* 1988). Also MRL-lpr/lpr mice that spontaneously develop an erosive arthritis show large numbers of these cells in their synovia in the absence of a significant lymphoid infiltrate, at least during the early stages of the disease (O'Sullivan *et al* 1985, Tarkowski *et al* 1987).

Large numbers of these cells in chronically inflamed synovium from humans show phenotypic characteristics of activation such as elevated expression of MHC class II (Athanasou *et al* 1988), Fc and complement receptors (Highton *et al* 1989, Broker *et al* 1990) and β2 integrins (Allen *et al* 1989). There are many potential factors that may be responsible for macrophage activation in joints (reviewed in Highton and Palmer 1994). These include activation by other cells such as lymphocytes and other macrophages, IC, bacterial cell wall products and activation resulting from viral infection. Activated macrophages may fulfill a variety of functions which include antigen presentation (Klareskog 1991), cytokine production (Arend and Dyer 1990) and the generation of a wide range of enzymes and inflammatory mediators (Johnston 1994). One important piece of evidence that macrophages play an important role in these diseases is the abundance of macrophage-derived cytokines, such as IL-1 and TNFα in the tissues of these joints as compared to the detectable quantities of T lymphocyte-derived cytokines (Firestein and Zwaifler 1990).

6.3. The role of DC in chronic synovial inflammation

DC are professional antigen-presenting cells (APC) (Steinman 1991) and have been identified in the synovial tissues from normal and diseased joints of humans and animal models of arthritis (March 1987, Dijkstra *et al* 1987, Holmdahl *et al* 1991). In humans, the number of DC in the synovial lining from patients with chronic inflammatory joint disease is reported to be higher than that from degenerative joint disease (Harding and Knight 1986, Iguchi *et al* 1986). DC are

present within lymphoid-rich areas of synovium and scattered throughout the interstitial areas (Iguchi *et al* 1986). The numbers of DC as a proportion of the large mononuclear cells in the synovial lining of human patients with inflammatory arthropathies has been reported to vary from 10 to 80% (Poulter *et al* 1983, Iguchi *et al* 1986). This variation could be accounted for by differences in criteria used to identify these cells, differences in disease states and the stage of the disease process that the tissues were examined. In SF from humans with RA, DC may form 1 to 5% of the mononuclear cell population (Zvaifler *et al* 1985, Summers *et al* 1995).

DC are very efficient accessory cells for T-dependent responses *in vivo* and *in vitro* and possess the unique ability of being capable of activating naive T cells (Steinman 1991). However, their role in the initiation and perpetuation of immune responses in synovium are unclear (March 1987). DC from SF of human patients with inflammatory joint disease act as highly efficient APC in recall responses and in a proportion of cases can cause powerful stimulation of autologous peripheral blood lymphocytes which is suggestive of antigen acquisition *in vivo* (Knight *et al* 1989, Stagg *et al* 1991). In non-lymphoid tissues the best characterised DC are Langerhans cells (LC) in skin. These are believed to capture antigen in the epidermis of the skin and transport it to the local draining lymph node via the afferent lymphatics (Silberberg-Sinakin *et al* 1976). Once in the lymph node these cells migrate to the paracortex and become interdigitating cells where they present their antigen to T lymphocytes. It is not known whether DC in synovial tissues function in a similar fashion, that is migration from the joint via the afferent lymphatics to the draining lymph node.

6.4. Other cell types

Fibroblasts are present in the synovial lining within the SLC layer and in the SI. Local proliferation of these cells contributes to the increased numbers of cells present in chronically inflamed synovium (Qu *et al* 1994) and they have the capacity to synthesise cytokines (Mourad *et al* 1992) and metalloproteinases

(McCachren 1991). Following stimulation *in vitro,* synovial fibroblasts can also act as APC (Boots *et al* 1994).

Mast cells are found in normal and diseased synovium from humans and animal models of arthritis (Godfrey *et al* 1984, Van den Broek *et al* 1988). Although these cells contain a wide range of preformed mediators of inflammation in granules (such as histamine, heparin and neutral proteases) and following activation can release cytokines such as TNFα and IL-4, their role in the pathogenesis of chronic inflammatory synovitis is unclear (Schwartz 1993).

Although neutrophils are a feature of acute inflammatory reactions, they are usually present in increased numbers in SF from chronically inflamed joints. Following activation neutrophils can release a wide array of inflammatory mediators which may contribute to tissue destruction in joints (Kitsis and Weissman 1991) and they have a limited capacity to produce cytokines (Cassatella 1995).

7. Mechanisms responsible for the attraction and entry of different cell populations in normal and inflamed joints

Although a large amount of information has been generated by characterising the cell populations at sites of chronic synovial inflammation in humans and animal models, the inflammation at these sites is not static but continually evolving. Studies of cell trafficking through chronic inflammatory sites in the skin of sheep have shown that the volume of cell traffic through these sites greatly exceeds the number of cells that are resident within the inflammatory site at any one time (Hay 1979). This is also likely to be true for chronically inflamed joints.

The process of cell trafficking through tissues can be divided into 3 phases; migration across endothelium, cell-cell and cell-matrix interactions within the tissue, and finally emigration via the afferent lymphatic vessels. Although some understanding has been obtained of the first 2 processes for joint tissues, very little is known regarding the emigration of cells from joint tissues.

The mechanisms controlling the entry of peripheral blood cells into normal and inflamed tissues have only recently been elucidated (Picker and Butcher 1992, Springer 1994). For neutrophils and monocytes a three step paradigm of leucocyte attachment and emigration through blood vessel endothelia has been proposed (Springer 1994). These steps include tethering and rolling of the leucocyte along the endothelium (mediated by selectins), activation of the cell, and finally adhesion strengthening and arrest (mediated by integrins) prior to transendothelial migration. Rather than being strictly sequential, these three steps are overlapping and heavily influenced by the production of chemoattractants within the tissues. For lymphocytes, the involvement of selectins in the initial adhesive interactions is unclear with some authors suggesting that P-selectin may mediate this process for some lymphocyte subsets (Luscinskas *et al* 1995), whilst others have suggested that integrins alone may be responsible for this process in some tissues (Berlin *et al* 1995). However, endothelial cell adhesion molecule-1 (ELAM-1) plays an important role in the extravasation of cutaneous lymphocyte antigen-expressing (CLA) T lymphocytes at sites of chronic inflammation in the skin (Picker *et al* 1991) demonstrating their importance in certain situations.

Because an understanding of the dynamics of cell trafficking through joints is incomplete without some knowledge of the factors responsible for the attraction of different cell populations into these tissues, these are briefly summarised below.

7.1. Neutrophils

The initial rolling of neutrophils along endothelium is mediated by selectins with firmer adhesive interactions mediated by leucocyte function-associated antigen 1 (LFA-1) interacting with intercellular adhesion molecule 1 and 2 (ICAM)(Bevilacqua 1993). Blood vessels expressing selectins and ICAM-1, -2 and -3 have been identified in the synovial lining from humans (Johnson *et al* 1993, Veale *et al* 1993, Szekanecz *et al* 1994). Additionally, *in vivo* blocking studies

using Mab to the β2 chain of LFA1 have been shown to reduce neutrophil entry into joints during AIA (Jasin *et al* 1992).

Chemokines responsible for attracting these cells into joint tissues include C5a, leukotriene B4 (LTB-4) platelet-activating factor (PAF), IL-8 (Koch *et al* 1991) and epithelial neutrophil activating peptide-78 (Koch *et al* 1994a). In AIA, elevated levels of PAF and LTB-4 have been demonstrated in SF shortly following induction of arthritis (Zarco *et al* 1992, Henderson and Higgs 1987) and decomplementation results in diminished numbers of neutrophils in affected joints (Rawson and Torralba 1967, DeShazo *et al* 1972). Although intraarticular injections of IL-1 have been shown to result in the accumulation of neutrophils in the joints of rabbits (Pettipher *et al* 1986) and mice (Van de Loo *et al* 1989) and IL-1 is generated during AIA (Henderson *et al* 1988), it is not a chemoattractant for neutrophils (Georgilis *et al* 1987).

7.2. Lymphocytes

Although vascular cell adhesion molecule 1 (VCAM-1) expression has been reported to be absent or present weakly on blood vessels in normal or inflamed synovium from humans (Van Dinther-Janssen *et al* 1991, Wilkinson, L. *et al* 1993), *in vitro* binding studies suggest that interaction between this molecule and its ligand, very late antigen 4 (VLA-4), are important for the extravasation of lymphocytes in chronically inflamed synovia (Van Dinther-Janssen *et al* 1991). Similar studies have also suggested that the interaction of ELAM-1 with its ligand may be of particular importance for the entry of lymphocytes into inflamed synovium (Postigo *et al* 1992). A recently discovered endothelial cell molecule that can mediate lymphocyte binding to endothelium, vascular adhesion protein 1 (VAP-1), has also been shown to be upregulated in inflamed synovium from humans (Salmi and Jalkanen 1992). However, the ligand for this molecule is not known.

Chemoattractants for lymphocytes that have been identified in synovial tissues from humans include IL-1 (Arend and Dyer 1990), IL-8 (Koch *et al* 1991), macrophage inflammatory protein-1α (MIP-1α)(Koch *et al* 1994b) and monocyte chemoattractant protein 1 (MCP-1)(Akahoshi *et al* 1993).

Of particular interest has been the suggestion that an endothelial cell recognition system may exist that controls the trafficking of lymphocytes into synovium that is distinctive from that which controls extravasation of these cells into other organ systems. For synovium, this was first suggested following the demonstration that certain lymph node or mucosal high endothelial venule (HEV)-specific B lymphoblastoid cells did not bind to synovial HEV in Stamper-Woodruff binding assays (Jalkanen *et al* 1986). Although it has been shown that certain B- and T-cell lines bind to synovial venules and not to lymph node or mucosal HEV (Salmi *et al* 1992), a molecule expressed exclusively by synovial endothelium has not been identified to date (Jalkanen and Salmi 1993). Neither has a molecule been identified that is expressed on lymphoid cells in the joint similar to that shown for CLA expression by memory T lymphocytes at inflammatory sites in skin (Picker *et al* 1990). *In vitro* blocking studies using Mab to VLA-4, VCAM-1, CD44, L-selectin, β1 and β2 integrins have shown complete or partial prevention of adhesion of lymphocytes from peripheral blood or SF to frozen sections of inflamed synovium from human patients with RA (Van Dinther-Janssen *et al* 1991). This suggests that a number of different lymphocyte-endothelial cell interactions may be responsible for the recruitment of cells into inflamed synovium. *In vivo* studies have been performed in humans with RA using Mab to ELAM (Kavanaugh *et al* 1994) and with Mab to ELAM and VLA-4 during the generation of AA in rats (Iigo *et al* 1991, Issekutz and Issekutz 1991). The results of the latter experiments also suggest that several different cell-endothelial cell interactions may be responsible for lymphocyte entry into inflamed joints.

7.3. Monocytes and DC

Relatively few studies have investigated the mechanisms whereby monocytes enter inflamed synovium in humans or animal models of arthritis. MIP-1α and MCP-1, which are chemoattractants for monocytes, have been shown to be generated in synovium from human patients with RA. *In vitro* studies have demonstrated that P-selectin may be the principal mediator of tethering of monocytes to inflamed synovium (Grober *et al* 1993) and *in vivo* studies of AA in rats have shown that antibodies to VLA-4 and LFA-1 are necessary to block monocyte migration into the inflamed joints of rats with AA (Issekutz and Issekutz 1995).

Very little is known of the factors that are responsible for the infiltration of DC at sites of inflammation anywhere in the body although LC have been shown to accumulate in the skin of humans following the intradermal injection of GM-CSF (Caux *et al* 1995). The recent demonstration that LC in the skin of humans express sialyl Lewis-x, which is a ligand for E-selectin, has suggested that this interaction may be involved in the recruitment of DC to skin (Ross *et al* 1994).

8. Interactions between leucocytes and components of the extracellular matrix in synovium

Following transendothelial migration, leucocytes will interact with the components of the ECM in synovium, including fibronectin, vitronectin, laminin and collagens. These interactions are mediated by cell adhesion molecules, in particular the members of the VLA family of proteins (Hemler 1990) and molecules such as CD44, the receptor for hyaluronate (Haynes *et al* 1991). The VLA proteins are heterodimers of a common β1 subunit together with one of six known α subunits (Hemler 1990). Recent attention has focused on the interactions of synovial T lymphocytes with fibronectin via VLA4 and 5 (Rodriguez *et al* 1992), and with laminin via VLA6 (Nikkari *et al* 1993). These interactions are of importance because they are likely to influence retention of T cells within the

synovial enviroment (Lazarovits and Karsh 1993) and the activation status and cytokine gene expression of these cells (Miyake *et al* 1993).

Monocyte/macrophage migration, differentiation and activation can also be influenced by interaction with the above-mentioned ECM proteins (Hemler 1990). The adhesive interactions of macrophages and fibroblasts to cartilage components is likely to play an important role in the invasion of pannus in erosive joint disease (Noyori and Jasin 1994).

9. Cell trafficking through normal and inflamed tissues

The different cell populations found in normal tissues can be broadly divided into 2 categories; those that are resident cells such as macrophages and fibroblasts, and those that have a natural tendency to emigrate via the afferent lymphatic vessels such as lymphocytes and DC. Although the appearance of these cells in joint tissues can be determined by sampling of synovial tissues, this gives little indication of the dynamics of migration into and emigration from these tissues. Most of our understanding of the kinetics of cell trafficking through normal and inflamed tissues have been determined from experimental studies performed on sheep, mice and rats (reviewed in Husband 1988).

Lymphocytes in peripheral blood and lymph form part of a large pool of lymphocytes that is continously recirculating between lymphoid and non-lymphoid tissues via the afferent and efferent lymphatic vessels. The advantage that the sheep has over rodent models of lymphocyte recirculation is its size. This allows individual afferent or efferent lymphatic vessels to be cannulated which enables the kinetics, phenotyping and antigen specificity of cells emigrating from individual tissues to be determined over extended periods of time. An underlying assumption is that there is economy and non-randomness in the recirculation of these cells such that different cohorts of cells of differing antigen specificites and developmental potential are directed through tissues where they are most likely to encounter antigen and fulfill their effector function.

The vast majority of lymphocytes in peripheral blood recirculate via lymphoid tissue, where they reside for approximately 18 to 20 hours before leaving via the efferent lymphatics (MacKay 1991). Radiolabelling studies have shown that the residency times of lymphocytes trafficking through chronically inflamed skin are similar to that of lymph nodes (Issekutz *et al* 1981). The majority of T cells in EL have a naive-type phenotype whereas the T lymphocytes draining non-lymphoid tissues in AL have a memory phenotype (MacKay *et al* 1990). This differential distribution of memory T cells trafficking through peripheral tissues ensures that more rapid responses are made if antigen is encountered in these tissues because proliferative responses to recall antigens resides within this population of T cells (MacKay 1991). Differences in recirculation pathways of naive and memory T cells can be accounted for by their surface expression of a number of CAM.

A central paradigm of lymphocyte migration studies is the existence of distinct migration pathways. The existence of these pathways was elucidated by cell-labelling studies. Lymphocytes in AL draining normal tissues (skin and gut) and those in EL, following labelling *in vitro* and reinjection back into the peripheral blood, were found at a higher frequency in the lymphatic compartment from which they were obtained compared to lymphatics draining other tissues of the body (Chin and Hay 1980, MacKay *et al* 1992b). It must be stated that these observations merely reflect preferences on the part of lymphocytes to recirculate through the same tissue and it is unclear whether this low level of recirculation is a reflection of antigenic stimulation or is truly constitutive (Picker and Butcher 1992). A similar propensity to recirculate through the same site has been shown for chronically inflamed skin (Chin and Hay 1980, Issekutz *et al* 1981) although it has not been demonstrated for other chronically inflamed tissues. The fact that labelled cells could be enriched by passing collected cells through nylon wool columns indicated that most of the recirculated cells were T lymphocytes (Abernethy and Hay 1988). However, other studies that have attempted to resolve this issue have shown mixed results with B lymphocytes from efferent lymph draining a peripheral

lymph node showing a similar propensity to recirculate compared to T cells, whereas this phenomenen was not observed for B lymphocytes draining EL from the gut (Reynolds *et al* 1988). The low proportion of B lymphocytes relative to T lymphocytes in AL draining peripheral tissues (MacKay *et al* 1988a) makes resolution of this issue considerably harder. These observations have since been extended by the demonstration that CD4+ and T19-expressing γδ T lymphocytes recirculate through lymph nodes with a greater efficiency than CD8+ T cells (Witherden *et al* 1990, Abernethy *et al* 1991).

The question also arises as to whether this propensity to recirculate through the same anatomic site is a feature of small lymphocytes or lymphoblasts. For T lymphocytes, there is general agreement that T lymphoblasts migrate in a tissue-specific manner (Hall *et al* 1977, Rose *et al* 1978) whereas for small T lymphocytes results have varied with some authors suggesting that these cells are responsible for tissue-specific recirculation (Hall *et al* 1977, MacKay *et al* 1992b) whilst others have found no evidence for this (Freitas *et al* 1977). Investigations in the sheep have shown that the propensity of lymphocytes to recirculate in a tissue-specific manner is a feature of small lymphocytes and lymphoblasts (Scollay *et al* 1976)

The role of antigen in the migration of lymphocytes through tissues has also been addressed. During secondary immune responses there is an early substantial increase in the blood flow to the tissue (Hay and Hobbs 1977). This begins within a few hours of challenge and is thought to be mediated by prostaglandins or other related products of arachidonic acid metabolism (Hay 1980). For lymph nodes, following secondary antigenic challenge there is a dramatic fall in the output of cells from the node which lasts one to two days and is thought to be caused by prostaglandins (PG), complement activation or γ-interferon acting singly or in combination (Hopkins *et al* 1981a, b, McConnell *et al* 1981). Antigen-specific T cells are not detectable in the efferent lymph for the first two to three days following antigenic challenge but then reappear after this period (Hay *et al* 1974).

This disappearance of antigen-reactive T lymphocytes is specific for the challenging antigen. It is unclear whether there is a corresponding loss of antigen-specific B cells from the lymph at this time. However, antigen may alter the migration of antigen-specific B cells through lymph nodes by incorporating them into germinal centres (Butcher 1986, Ponzio and Thorbecke 1988). Studies in mice have confirmed that memory B lymphocytes can accumulate in lymph nodes draining sites of antigen deposition (Ponzio and Thorbecke 1988). Antigen has also been shown to influence the localisation of antigen-specific plasma cell precursors in the intestine of rats (Husband 1982).

With the development of Mab to ovine B and T lymphocyte subsets the understanding of cell recirculation has been further refined. Several authors have investigated the effects of secondary antigenic challenge of lymph node and skin to determine whether there are subset-specific changes in the migration of lymphocytes through these sites. For lymph node, following secondary antigenic challenge, there is a biphasic emigration from the node with a peak in the percentage of CD4+ T lymphocytes between day 1 and 2 post-antigenic challenge followed by a peak of CD8+ T lymphocytes between day 4 and 5 (Bujdoso *et al* 1989a, MacKay *et al* 1992a). Additionally, the throughput of memory T lymphocytes is considerably increased over this time period and VCAM-1 expression by blood vessel endothelia within the node is up-regulated (MacKay *et al* 1992a). Previous studies have shown that antigen-reactive cells are not detectable in efferent lymph during the first 2 to 3 days following secondary antigenic challenge of the node, which corresponds to the peak lymphocyte output of the node, after which time antigen-responsive cells reappear (Hay *et al* 1974, Hay and Morris 1976). Whether a similar phenomenen occurs during secondary antigenic challenge of tissues other than lymph node has not been established. The only studies that have been performed to characterise lymphocytes draining chronic inflammatory sites, since the widespread availability of ovine-specific Mab, have investigated changes either during the induction phase of subcutaneous granulomas

or during the more chronic stages of these lesions (Kimpton *et al* 1990, MacKay *et al* 1992a). These showed that 5 to 6 weeks following induction of the granuloma lymphocyte trafficking through the lesion was markedly elevated compared to normal skin and that there were significant alterations in the percentages of the T lymphocyte subsets in the AL draining these lesions compared to lymph draining non-inflamed skin (Kimpton *et al* 1990).

Although less well studied than the cellular changes in lymphatic vessels, a variety of soluble factors have been detected in AL draining chronic inflammatory sites in the skin of sheep. These include PGE_2, $PGF_{2\alpha}$ (Johnston *et al* 1980), phospholipase A2 (PLA2)(Vadas and Hay 1982) and IL-1 has been detected in AL draining normal skin in humans (Plachta *et al* 1988). With the exception of IL-2-like activity that has been detected in AL and EL following secondary antigenic challenge of skin (Budjoso *et al* 1990), there are no reports of the levels of cytokines in the lymph of sheep.

The best characterised of the above-mentioned mediators are the prostaglandins. The predominant prostaglandin in AL is PGE_2. This is present in lymph complexed to albumin (Johnston *et al* 1980, Hopkins *et al* 1981b) and together with PLA2 is believed to be the cause of the increased blood flow to the lymph node following antigenic challenge (Moore *et al* 1980).

10. Lymphatic drainage from joints

Afferent lymphatic vessels have been identified in synovium from several species. Davies (1946) demonstrated the existence of these vessels in synovium of the distal limb joints of cattle by injecting diluted ink directly into the synovium. Throughout most of the synovium, lymphatics exist as part of a freely anastomosing plexus with numerous blind-ending smaller tributaries that frequently terminate in lacuniform enlargements. These vessels were reported to be fewer in number and less superficial than the most superficial plexus of capillaries that is found immediately beneath the synovial lining cells. The lymphatic plexus becomes

more attenuated in the more fibrous areas of synovium, towards the chondrosynovial junctions and were reported to be absent from synovial villi. From this network of lymphatics, collecting trunks pass in small groups alongside the main blood vessels and eventually enter the local draining lymph nodes. Afferent lymphatics have also been identified in human synovium following arthrography (Kormano and Makela 1978) and in tissue sections using a combination of morphological criteria and immunohistochemical characteristics (Wilkinson and Edwards 1991). Interestingly, afferent lymphatic vessels could not be identified in the synovial lining from the joints of humans with RA (Wilkinson and Edwards 1991) raising the possibility that these structures may be destroyed as a consequence of the chronic inflammatory process. Alternatively, it is possible that the expression of the molecules used to identify these vessels is altered in inflamed synovium. Nothing is known regarding the expression of CAM by lymphatic endothelia although for synovium from humans it has been reported that they do not express VCAM, ICAM or ELAM (Wilkinson and Edwards 1991). Consequently, nothing is known of the nature of the interactions between cells and lymphatic endothelia.

Although cell emigration from joints via the afferent lymphatics has never been confirmed, it has been established that lymphatic drainage is the major route for removal of proteins from articular tissues (Bauer *et al* 1933). Studies of clearance of radiolabelled albumin from joints have shown that protein diffusion is increased in inflammatory versus non-inflammatory joint disease in humans (Wallis *et al* 1987).

In an effort to address the issue of cell trafficking through inflamed joints, Thorpe *et al* (1992) generated a CICIA in sheep. Although they were not able to characterise the cellular components of the afferent lymph draining the inflamed joint, they did demonstrate elevated levels of antigen-specific antibody in the AL following induction of the arthritis.

Several studies have investigated the removal of particulate antigen from joint cavities (Key 1926, Adam 1966). Both showed that colloidal carbon particles were rapidly ingested by resident and invading macrophages following its injection into the joint cavity, and Key (1926) demonstrated that macrophages laden with carbon were present in the lymph node draining the joint. Very little free carbon was present in the joint cavity after about 5 days following its injection but the amounts present in the lymph node increased up to 2 weeks following injection suggesting that this accumulation may have been due to carbon arriving in the node associated with cells rather than by diffusion from the joint.

Aims of this thesis

Our understanding of the pathogenesis of inflammatory joint disease in sheep is very unclear. Because of their larger size compared to laboratory animal models of arthritis and the ease with which synovial tissues can be obtained, they are a good host species for studying the biology of normal and diseased synovium. Additionally, they provide the potential to investigate aspects of the dynamics of synovial inflammation, using lymphatic cannulation techniques, that cannot be studied in other species.

The first aim of this thesis was to characterise synovial tissues from normal sheep, comparing different stages of development from the foetus to the adult. Secondly, lentiviral arthritis in sheep provides a natural model of chronic inflammatory arthritis associated with a viral infection. Characterisation of this disease, particularly in its early stages, may provide a valuable insight into the pathogenesis of arthritic diseases associated with these agents that may be of relevance to the understanding of arthritis in other species infected with these viruses, including humans.

The use of lymphatic cannulation techniques performed in sheep over the past 30 years have provided us with a unique insight into the dynamics of immune responses and of the characteristics of cell trafficking and recirculation through

normal and inflamed tissues. At present, the characteristics of cell trafficking through normal or inflamed joints is a mystery. An understanding of this process would provide us with a broader view of the pathogenesis of chronic synovial inflammation. Hence, the third major aim of this thesis was to generate a model of chronic synovial inflammation, based on AIA that has been described in other species, that would allow the dynamics of cell trafficking through an inflamed joint to be investigated.

CHAPTER 2

Materials and methods

1. Materials

Unless otherwise stated, all products were purchased from SIGMA, Chemical Co., Poole, Dorset.

2. Experimental sheep

2.1. Sheep used for the study of developmental changes in synovium (Chapter 3)

Lambs of varying ages were obtained from a flock maintained at the Moredun Institute, Edinburgh. Foetal lambs from late in gestation were obtained from the Edinburgh Meat Plant, Gorgie, Edinburgh, and from the MRC unit, Marshal building, Roslin, Midlothian. Foetal age was estimated from the formula:

Age (days) = 2.1 x [(crown-anus length) + 17] (Arthur *et al* 1983)

(crown-anus length measured in cms)

2.2. MVV-infected and control sheep (Chapter 4)

A flock of sheep naturally-infected with MVV was purchased by the Department of Veterinary Pathology, Edinburgh in 1989 and maintained at the Royal (Dick) School of Veterinary Studies Field Station, Bush, Roslin, Midlothian. Infection was confirmed serologically by agar gel immunodiffusion test (AGID). Because of poor record keeping by the flock's previous owner the precise age of individual animals was not known. Although some indication of age can be obtained by inspection of the dentition (St. Clair 1975), this can be affected by feeding management and therefore is not wholly reliable. All MVV-infected sheep used in this study were adult females whose age was estimated to be at least four years, based upon their dentition and duration of ownership by the Department.

Because of the difficulty in obtaining precise age- and breed-matched control animals, female sheep were purchased from the Moredun Institute, Edinburgh, that were of similar size and conformation to the MVV-infected sheep. These sheep were female, aged at least four years and were seronegative for MVV by AGID.

2.3. Sheep used for the generation of AIA (Chapters 5 and 6)

All sheep used in these experiments were female, 1 to 2 years of age and of the Scottish Blackface breed. These sheep showed no clinical signs of joint abnormalities at the time of purchase (Macauley Land Use Research Institute, Morebattle, Roxburghshire).

2.3.1. Generation of AIA

Sheep were immunised to ovalbumin (OVA, grade V) in complete Freunds adjuvant (CFA). 1mg/ml OVA in sterile PBS was emulsified in an equal volume of CFA and injected subcutaneously along the flank (maximum of 1ml injected in four different sites). This was repeated with OVA emulsified in incomplete Freund's adjuvant (IFA) two weeks later. Two to three weeks after immunisation, peripheral blood (PB) samples were taken and the serum assessed for anti-OVA IgG antibodies by enzyme-linked immunosorbent assay (ELISA)(section 12.1).

Prior to the intraarticular injection of OVA, sheep were sedated with 0.12 mg/kg xylazine (Rompun, Bayer plc, Bury St. Edmunds, Suffolk) administered intramuscularly. With the sheep held on its hauches by an assistant, the hair around both radiocarpal (RC) joints was clipped and the skin cleaned with diluted povidone-iodine solution followed by surgical spirit. 0.5mg OVA (0.5ml of a 1mg/ml solution of OVA in endotoxin-free 0.9% sterile NaCl solution passed through a 0.22μm millipore filter) was injected into the right RC joint and 0.5ml of the 0.9% NaCl solution was injected into the left RC joint. These injections were made with a 23G, 1" needle attached to a 1 ml syringe and SF was always withdrawn into the syringe before injection. Sheep with AIA were maintained in small groups in strawed pens and fed a diet of hay, commercial meal and *ad lib* water.

3. Clinical assessment of joint disease

3.1. MVV-infected sheep

Following careful palpation of the joints of the fore- and hindlimb, individual MVV-infected sheep were defined as having clinical arthritis if they had synovial effusions or palpable thickening of the soft tissue structures of any joint. The latter was graded as mild, moderate or severe. This assessment concentrated on the joints of the distal limb because of the known predisposition of these joints to develop arthritis in MVV-infected sheep and because of the difficulty in evaluating the shoulder and hip joints due to the depth of soft tissues overlying these joints. Although lameness was present in some arthritic sheep, lameness *per se* was not taken to be indicative of joint disease. Other criteria that are routinely used in the assessment of joint disease in other species such as pain or crepitus elicited by manipulation were generally found to be of limited value in the evaluation of joint disease in sheep. Sheep showing no signs of lameness and with no palpable thickening of the soft tissue structures of their joints or synovial effusions were defined as non-clinically arthritic. This category included sheep with normal joints and those with subclinical arthritis.

3.2. Sheep with AIA

The severity of joint enlargement was determined from calculation of the carpal:metacarpal (C:MC) ratio. These measurements were made at various time points using a flexible measure at the level of the accessory carpal bone for the carpal measurement and at the mid-metacarpal level for the metacarpal measurement. Because the latter remains constant following generation of AIA, the ratio is a measure of the severity of joint enlargement.

4. Post mortem examination of joints

The majority of post mortem examinations of sheep joints in this study were performed on the RC and tibiotarsal (TT) joints. Enlargement of these or other joints was recorded as mild (1+), moderate (2+) or severe (3+). The procedure below was

followed for all RC and TT joints. Other joints were rarely examined but when they were, a similar procedure was used to inspect the articular surfaces and associated soft tissues.

Following removal of the skin from around the joint, a transverse incision was made through the tendons on the anterior aspect of the joint at its most proximal level. A transverse incision was then made through the joint capsule close to its attachment on the distal radius for the RC joint, and distal tibia for the TT joint. The joint capsule and associated synovial lining were reflected distally to expose the articular cartilage of the bones comprising each articulation. Several different lesions of the articular cartilage were identified. These were:

1. Small full or partial thickness cartilage defects on the articular surfaces (up to 5 or 6mm across), superficial abrasions and minor fissuring. These were recorded if present but not graded.
2. Erosions of articular cartilage and underlying subchondral bone associated with the invasion of pannus or adjacent to areas of inflamed synovium. These were graded as mild (1+), moderate (2+) or severe (3+), depending upon their severity.

Any changes in the appearance of the synovial lining were recorded. Synovitis, characterised by an increase in the thickness of the synovial lining and discolouration, was graded as mild, moderate or severe. To limit any effects of variation in histological appearance of synovium from different regions within the joint, biopsies were always obtained from the same location in the RC and TT joints. From the RC joint, synovium and joint capsule were dissected from the inner aspect of the tendon of the *extensor carpi radialis* muscle that crosses the anterior aspect of the RC joint in the midline (Getty 1975). Synovium from the TT joint was always obtained from adjacent to the tendon of the *cranial tibial* muscle (Getty 1975).

5. Processing of synovium

Each biopsy of synovial lining was divided in two and one portion was fixed in 10% buffered formol-saline and the remainder was snap frozen in isopentane cooled

with dry ice. These blocks of tissue were approximately 5mm x 10mm and 4mm in depth (The depth was greater when the tissue was hypertrophied). Frozen tissue was then mounted on cork blocks with OCT compound (Miles Inc., Elkhart, IN 46515, USA) with an orientation to ensure that sections would subsequently be cut at 90^0 to the tissue surface. Frozen tissues were stored at -70^0C. Histological sections from the formol-fixed tissue were stained with haematoxylin and eosin (H+E), methyl pyronin green (Plasma cells), Massons trichrome blue (Fibrin) and toluidine blue (Mast cells). These routine staining procedures were performed by the technical staff of the Department of Veterinary Pathology, Veterinary Field Station, Bush, Midlothian.

6. Investigations of the synovial lining

6.1. Synovial histology

Histologically, the synovial lining can be divided in to two layers. Different authors vary in the terms that they use to describe these layers and the cells that comprise them. In this thesis, the layers of cells immediately adjacent to the joint space is referred to as the synovial lining cell layer and the cells comprising this layer are referred to as synovial lining cells (SLC)(Figure 1a). The tissue beneath these cells is referred to as the subintima (SI)(Henderson and Pettipher 1985). These terms are used to define the location of cells and do not imply distinct functional characteristics. Additionally, there is no basement membrane separating SLC from cells in the SI (Henderson and Edwards 1987) and their differentiation can be problematic. Lymphocytes, plasma cells and neutrophils were identified from sections of formol-fixed tissue by their conventional morphological critera. With the exception of endothelial cells, problems were encountered with distinguishing other cell types known to be present in synovium. These included macrophages, fibroblasts, DC and mast cells. Because conventional histology does not allow these cells to be differentiated, they were refered to as large mononuclear cells (LMN)(Edwards *et al*

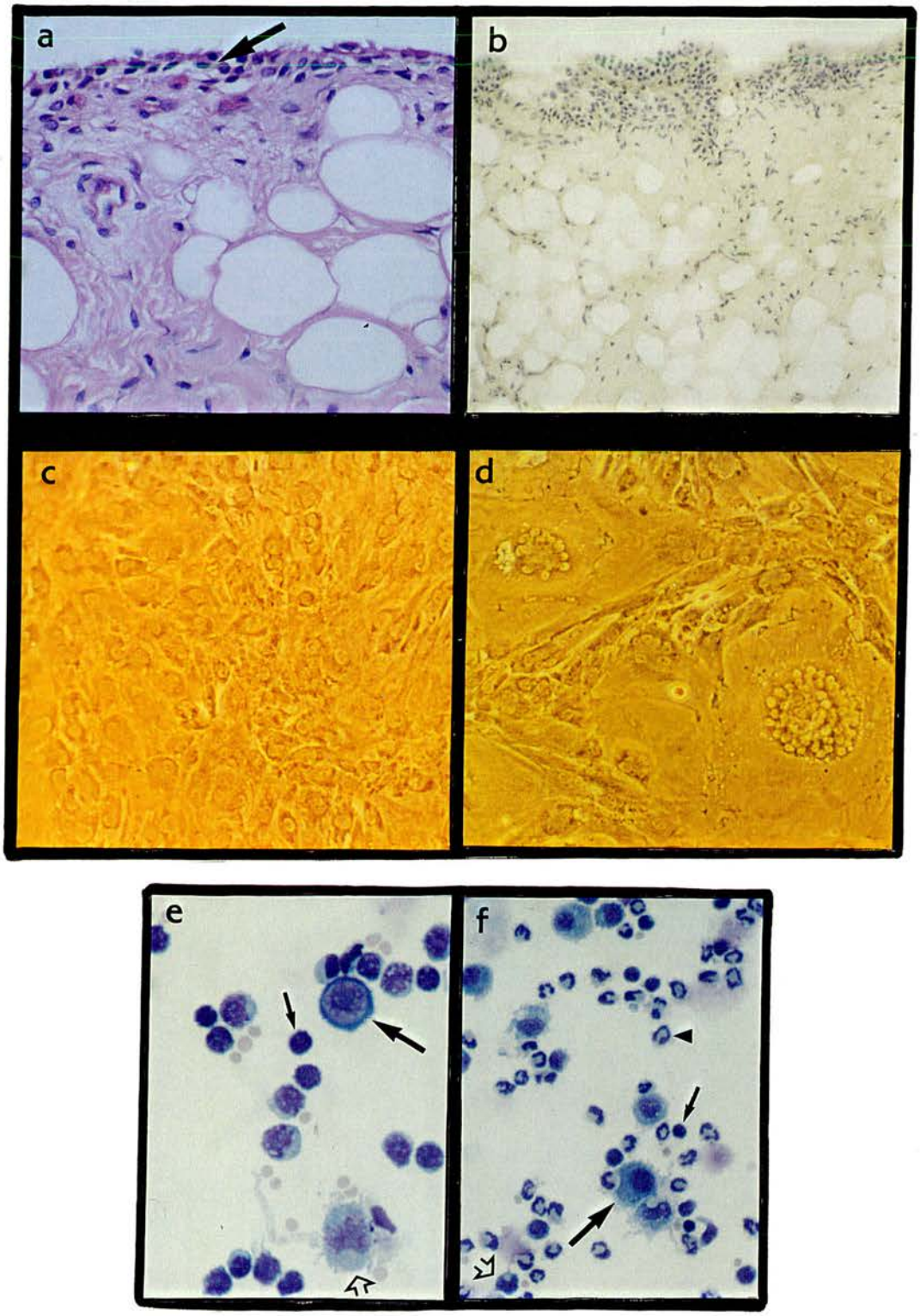
Figure 1a. Histological appearance of the synovial lining from the RC joint of a control sheep (H+E, original magnification x390).
The arrow indicates the SLC layer.

Figure 1b. Representative cryostat section of synovial lining labelled with Mab VPM53 (anti-Campylobacter) as a negative control (Immunoperoxidase technique, original magnification x156).

Figure 1c and 1d. Isolation of MVV on monolayer cultures of ovine synovial membrane cells.
Several syncytia are present in the MVV-infected monolayers (d) in contrast to the non-infected cultures (c).

Figure 1e and 1f. Cytospin preparations of AL (e) and SF (f)(Leishman's stain, original magnification, x390).
The cells identified in AL were DC (open arrow), lymphocytes (small arrow) and large basophilic lymphoblasts (large arrow). Cells identified in SF were synoviocytes (large arrow), lymphocytes (small arrow), macrophages (open arrow) and neutrophils (triangle).

Figure 1



1988). All histological sections were evaluated using a Leitz Ortholux II microscope using a x25 objective (Final magnification of x390).

6.2. Immunohistology of synovium

Sections 6 to 8μm thick were cut on a Shandon cryostat and placed on slides previously coated with poly-D-lysine (Vectabond; Vector Labs., Bretton, Peterborough). Sections were air-dried overnight and then fixed in cold acetone (4^0C, 5 mins). Non-specific Fc receptor binding of monoclonal antibody (Mab) was blocked by incubation in PBS containing 10% normal sheep serum (20 minutes, room temperature). For the following stages, reagents were obtained from a commercially-available immunohistology kit (Immustain; EuroDPC, Whitney, England) and all incubation steps were performed in a humidity chamber at room temperature, unless otherwise stated. Mab were applied to the sections (100μl per section) as hybridoma supernatants, or ascitic fluid whose optimal dilution for maximum intensity of staining with minimal background levels had been pre-determined. As controls, sections were incubated in normal mouse serum (diluted 1:500 in PBS) or an isotype-matched Mab, irrelevant to the study. Following incubation for 2 hours the sections were washed in two changes of PBS containing 0.01% sodium azide (PBS/Az)(10 minutes per wash). A biotin-labelled anti-mouse immunoglobulin (Ig) was then applied to the sections for 45 minutes followed by one wash in PBS/Az (5 minutes). A peroxidase-labelled streptavidin was then applied for 45 minutes. Following one wash in PBS/Az (5 minutes), the sections were incubated in pre-warmed glucose oxidase solution (PBS containing 0.2% β-D-glucose and 6u/ml glucose oxidase)(Koller *et al* 1986) for 15 minutes at 37^0C. Following one wash in PBS/Az (5 minutes) the sections were incubated in diamino-benzidine tetrachloride (DAB) solution for 6 to 8 minutes after which the slides were washed in running tap water for 15 minutes. Sections were lightly counterstained in haematoxylin, blued in lithium carbonate and then passed through a series of graded alcohols and a histological clearing agent prior to mounting of coverslips.

Positively-stained cells were identified by the deposition of DAB. Control sections were uniformly negative for DAB staining (Figure 1b).

6.3. Non-specific esterase staining for macrophages (Hudson and Hay 1989)

Sections 6-8μm thick were cut on a cryostat, air-dried and fixed in a mixture of cold 45% v/v formaldehyde, 25% v/v acetone in 0.1M phosphate buffer (pH 6.6) for 40 seconds. The sections were then washed in water and incubated in 0.025% α-naphthyl butyrate developed in 0.067M phosphate buffer pH5.8 (containing 0.12% w/v sodium nitrate and 0.12% w/v pararosaniline) for 40 minutes at 37^0C. The sections were washed in running water, counterstained in 2% methylene green for 10 minutes before being passed through graded alcohols and a histological clearing agent. Positively-stained cells were identified by the presence of a brown precipitate.

6.4. Morphometric analysis of synovium

Semiquantitive and quantitiative methods were used to assess the histological and immunohistological characteristics of synovium in this thesis.

6.4.1. Histological scoring of sections from formol-fixed tissues

For this purpose a semi-quantitative scoring system was adopted. This involved assessment of the number of layers of cells comprising the SLC layer, and in the numbers of lymphoid cells that were identifiable in the SI. An increased thickness of the SLC layer was graded as mild (1+; 3 to 4 cells thick), moderate (2+; 5 to 7 cells thick) or severe (3+; more than 7 cells thick). For sections showing variation in the numbers of layers of cells comprising this SLC region, a score was ascribed that reflected the predominant appearance for that section. A minimum area of 10 fields of view using a x25 objective were assessed on each section.

The intensity of any lymphoid infiltrate was also graded as mild (1+, small numbers of diffusely scattered cells), moderate [2+, increased numbers of diffusely scattered cells with less than 3 focal or perivascular aggregates (defined as at least 50

cells) of lymphoid cells per section] or severe (3+; large numbers of lymphoid cells with 3 or more perivascular or focal aggregates per section). Although some authors have described more elaborate scoring systems including assessment of vascularity, fibrin deposition and fibrosis (Rooney *et al* 1988, Cooke 1988), these features were noted but not scored in this study.

6.4.2. Semi-quantitiative evaluation of synovial immunohistology.

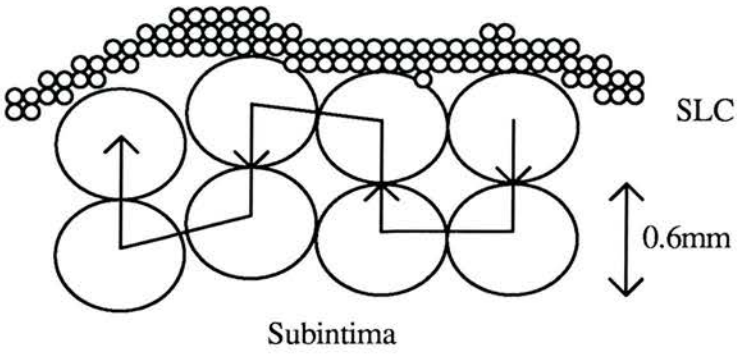
This grading system was used for scoring the expression of some non-B and non-T lymphocyte-specific antigens (Chapter 3). The SLC and SI layers were evaluated separately. Positively-stained cells were defined as being absent (-), present occasionally (1+), or in moderate (2+) or large numbers (3+). If all cells were positively-stained the section was graded 4+.

6.4.3. Quantitative evaluation of synovial immunohistology

6.4.3.1. Lymphocyte densities

Positively-stained cells within a defined area of synovium (minimum area counted was $2mm^2$)(Figure 2) were counted, to a depth of approximately 1mm from the SLC (this distance was approximately twice the diameter of the projected field of view using a x25 objective). This was performed at x390 magnification using a graticle eyepiece and identical areas of tissue were analysed on sequential sections stained with the relevant Mab. The results were expressed as the number of positively-stained cells/mm^2. Lymphocytes were present at a depth of greater than 1mm from the SLC and therefore the results reflect the densities of these cells in the superficial areas of synovium. The size of the minimum area counted has previously been shown to be the minimum that is representative for determining cellular density from cryostat sections of synovium from humans with inflammatory and non-inflammatory arthropathies (Kennedy *et al* 1988a, b). Although it was not always possible to analyse an uninterrupted area of synovium, care was taken to ensure that the same areas were analysed from sequential sections. The areas of synovium chosen

Figure 2. Evaluation of T lymphocyte densities in the synovial lining



Positively-stained cells were counted with the aid of a graticle field within the areas delineated by the field of view obtained with a x25 objective. Cells within a minimum of 8 fields of view were counted in this fashion (approximating to $2mm^2$)

for quantitation were largely determined by the presence of areas on sequential sections from which counting was considered to be possible.

6.4.3.2. MHC class II expression

The proportion of positively-stained SLC was determined by counting 500 adjacent cells on sequential sections stained with Mab to MHC class II (DRα- and DQα-chains). This was performed with the aid of a graticle eye-piece. The areas chosen for counting were selected on the basis that staining quality on sequential sections was adequate to allow this quantitiation to be performed.

The proportion of MHC class II-expressing cells in the SI was determined by counting 500 cells (excluding blood vessel endothelia) within a defined area of the SI to a depth of no greater than approximately 0.6mm (diameter of the projected field of view using a x25 objective) from the SLC. No attempt was made to differentiate different cell types during this quantitation or between different intensities of staining. Identical areas of synovium were analysed on sequential sections stained with the Mab.

6.5. Isolation of MVV from synovium

Synovium obtained at post mortem was collected into universal containers containing RPMI medium. Using flame-sterilised instruments in a biological hood, synovium was teased from the underlying joint capsule and placed in 25cm^2 tissue culture flasks with Dulbecco's modified Eagle's medium (DME; Gibco Biocult, Uxbridge, Middx.) containing 5% FCS (Flow Labs., Herts), 100i.u./ml of penicillin and streptomycin. These were maintained at 37^{0}C, 5% CO_2 until the cells that had grown out from the tissue were nearly confluent. Cells were then transferred between flasks following a brief incubation in 0.05% w/v trypsin in PBS, and maintained until characteristic syncytia were observed (Figure 1c and 1d). At this stage the cell cultures were transferred to petri dishes containing glass coverslips. Following adherence and the observation of syncytia, the coverslips were washed twice in

Hank's balanced salt solution and fixed in 0.15M sodium chloride in 80% acetone (2 minutes, 4^0C). The cover slips were dried with a hairdrier and then incubated in PBS containing 2% sheep serum and 0.01% Tween 20 (30 minutes, 20^0C). Following one wash in PBS containing 0.01% Tween 20, coverslips were incubated in Mab to the MVV core protein P15 (1:5000 dilution in PBS)(Table 1) for 1 hour (room temperature). Control coverslips were incubated in normal mouse serum (1:500 dilution). They were then washed 3 times in PBS/Tween and incubated in a fluorescein-conjugated anti-mouse IgG (diluted 1:100 in PBS) for 60 minutes. Following 3 washes in PBS/Tween the coverslips were mounted on glass slides with glycerol diluted with PBS (9 parts glycerol: 1 part PBS) and viewed under a Leitz fluorescence microscope. Positive fluorescence of syncytia confirmed the presence of MVV.

7. Collection of body fluids and cytological analysis.

7.1. Peripheral blood (PB)

PB was obtained by venepuncture of the jugular vein. When serum was required, PB was collected into glass universal containers. For purposes of flow cytometry, PB was collected into plastic tubes containing heparin (10i.u./ml final concentration)(Heparin sulphate; Leo laboratories, Princes Risborough, Bucks.). For the purposes of T lymphocyte proliferation assays, defibrinated PB was obtained by agitation of PB collected into glass Universal containers containing glass beads.

7.2. Afferent lymph

A total nucleated cell count (TNCC) was obtained using a modified Neubauer haemocytometer following dilution in 1% Trypan blue solution to determine cell viability. Following centrifugation (1000rpm, 5mins), supernatants were aliquoted and stored at -70^0C. Cells were resuspended in PBS containing 2% BSA, 0.1% Az and 1 i.u./ml heparin and cytospin preparations made in a Shandon cytocentrifuge (800 rpm, 10 mins). These were stained with Leishmans solution and differential cell counts

were made by counting 400 cells using a graticule eye-piece. The following cell types were identified and quantitated in AL (Figure 1e):

1. Neutrophils.

2. Lymphocytes.

3. Large basophilic lymphoblasts.

4. DC. These were identified by their size, convoluted nuclear morphology and cell processes.

Eosinophils and cells undergoing division were occasionally identified but their numbers were not quantitated.

7.3. Synovial fluid

SF was aspirated from joints at post mortem or using an aseptic technique from living sheep either under general anaesthesia (immediately prior to biopsy of the synovial lining; section 14.2.) or following sedation (details in section 2.3.1). SF was aspirated with a 21G needle attached to a 2 or 5ml syringe and collected in to 1.5ml plastic eppendorf tubes containing 50µl of PBS containing 25i.u. hyaluronidase (Grade V), and 0.5 i.u. heparin sulphate. After 1 minute of gentle rotation the sample was placed on ice until further analysis. SF grossly contaminated with blood was discarded.

TNCC were performed in the same way as for AL. Cytospin preparations of SF were made following dilution in PBS containing 2% BSA and 1i.u./ml heparin sulphate or following pelleting (10 mins, 500rpm) and resuspension in this medium, when the TNCC was low (under $5x10^5$/ml). SF supernatants were aliquoted and stored at -70^0C.

Detailed analysis of cytospin preparations has resulted in the identification of a large number of different cell types in SF from humans (Freemont and Denton 1991). A classification system was devised based upon that used for the evaluation of SF from humans. The following cell types were identified and quantitated in ovine SF (Figure 1f):

1. Neutrophils.

2. Lymphocytes.

3. Macrophages. These were identified by their convoluted nuclear morphology and a nucleus: cytoplasm ratio of approximately 30 to 60%.

4. Synoviocytes. These were recognised by the rounded appearance of their nucleus which was usually eccentrically placed, and a nucleus:cytoplasm ratio of less than approximately 40%.

Other cell types were noted if present but were not quantitated. The differentiation of certain cell types such as lymphoblasts and smaller synoviocytes was, on occasions, difficult. Although specialised stains and immunocytochemistry can be used to differentiate these cell types, this usually relies upon multiple cytospin preparations being made from any given sample. These were not performed because the volume and cell concentration of SF was often low and additionally, moderate numbers of cells were usually required for characterisation of the cell populations by flow cytometry. Hence, the results reflect the limitations of conventional light microscopy in allowing differentiation of cell types.

8. Cell separation procedures

8.1. Isolation of mononuclear cells from PB and SF

10mls of defibrinated PB was mixed with 30mls of sterile PBS (pH 7.4) in a 50ml sterile plastic tube and centrifuged (3000rpm, 20 minutes, 20^0C) over 10mls of Lymphoprep (Nycomed, Pharma AS, Oslo, Norway). Cells at the interface were removed and washed 3 times in RPMI containing 2% FCS. Cell viability was determined by 1% Trypan blue dye exclusion test.

Essentially the same procedure was followed for the isolation of mononuclear cells from SF except that following aspiration, SF was mixed with 5mls of RPMI containing heparin (1i.u./ml). The cells in SF were washed three times in RPMI containing 2% FCS and heparin (1i.u./ml) before being resuspended in 2mls of this

medium and centrifuged over 5mls Lymphoprep (conditions as above). The interface cells were then treated in the same way as cells from PB.

8.2. Isolation of DC from AL

Cells in AL were washed twice in RPMI containing 10% FCS. These were resuspended in 3mls of this medium and centrifuged (2000rpm, 15 minutes, 4^0C) over 14% Metrizamide (Nycomed, Pharma AS, Oslo, Norway) made up in the above medium (filter sterilised through a 0.22μm millipore filter). Cells at the interface were collected and washed twice in the above medium.

9. Monoclonal antibodies

The Mab used in this thesis are shown in Table 1. Unless otherwise stated they were generated in the Department of Pathology, Royal (Dick) School of Veterinary Studies, Edinburgh. Not all of these Mab were available at the beginning of this period of study.

10. Cell and protein labelling procedures

10.1. Biotin labelling of protein

Immunoglobulin (Ig) was purified from ascitic fluid by precipitation of unwanted proteins with 1.0M acetic acid and caprylic acid. Precipitated proteins were removed by microfuging for 15 to 30 minutes and the supernatant was dialysed extensively into PBS over 72 hours. The protein concentration was then adjusted to 1 mg/ml in 0.1M $NaHCO_3$ (pH 8.4) buffer solution. Biotin ester (2mg/ml in DMSO) was added to give a biotin:protein ratio of 75μg biotin:1mg protein. Following continuous rotation for four hours the solution was extensively dialysed against PBS containing 0.1% Az for 24 hours. The protein concentration was then determined on an Ultrospec 4050 spectrophotometer and aliquots stored at -70^0C. Confirmation of biotinylation was performed by flow cytometry.

Table 1. Monoclonal antibodies used in this thesis

Mab	Specificity	Isotype	Reference
ST4	CD4	G2a	Maddox *et al* 1985
SBUT8	CD8	G2a	Maddox *et al* 1985
CC15	T19[a]	G2a	MacKay *et al* 1989
86D	γδ TCR	G1	MacKay *et al* 1989
DU2104	pan B lymphocyte	M	MacKay *et al* 1992b
36F	CD2	G2a	MacKay *et al* 1988a
73B	CD45RA	G1	MacKay *et al* 1990
ILAIII	IL2r (α-chain)	G1	Naessens *et al* 1992
VPM54	MHC class II (DRα)	G1	Dutia *et al* 1990
VPM37	MHC class II (DRβ)	G1	Dutia *et al* 1990
VPM36	MHC class II (DQα)	G1	Dutia *et al* 1990
VPM41	MHC class II (DQβ)	G1	Dutia *et al* 1990
SW73.2	pan MHC class II	G (rat)	Hopkins *et al* 1986
SBU-T6	pan CD1	G1	MacKay *et al* 1985
F10-150-39	LFA-1	G1	MacKay *et al* 1990
L180-1	LFA-3	G1	MacKay *et al* 1990
Pgp1	CD44	G1	MacKay *et al* 1988b
HP1/2*	VLA4 (α-chain)	G1	Sanchez-Madrid *et al* 1986
GoH3*	VLA6 (α-chain)	G1	Sonnenberg *et al* 1988
DU129	L-selectin	G1	Spertini *et al* 1991
TS2/16*	β1 integrin	G1	MacKay *et al* 1992b
VPM63[b]	FcγRII	G1	Gupta 1994
VPM65[c]	CD14	G1	Gupta 1994
OM1	CD11c	G1	Gupta *et al* 1993
P15	MVV core protein P15	G	Houwers 1988
VPM53	*Campylobacter* sps	G1	McOrist *et al* 1987

* denotes Mab with specificity for human antigens which are cross-reactive with sheep tissues (MacKay *et al* 1992a).

[a] T19 is a molecule expressed exclusively on γδ T lymphocytes in cattle and sheep. The function of this molecule is unknown.

[b] VPM63 immunoprecipitates a molecule of 40-42 kD. Affinity purified antigen is recognised by anti-bovine FcγR peptide antiserum (Gupta 1994).

[c] VPM65 immunoprecipitates a molecule of 55 kD. The molecule is associated with the cell surface via glycosyl phosphatidyl inositol linkage and the Mab recognises the same antigen as the anti-human CD14 Mab TVK4 (Gupta 1994).

The biotinylation procedure for proteins [OVA and human serum albumin (HSA)] was identical to that for purified Ig. Confirmation of biotinylation was performed by ELISA.

10.2. Fluorescein labelling of protein

20 mg of protein was dissolved in 1ml of Na_2CO_3 (0.25M, pH 9). To this was added 100µl fluorescein isothiocyanate (FITC) dissolved in DMSO (1mg FITC/ 100µl DMSO). Following incubation for 1 hour (20^0C) the solution was passed through a Sephadex G10 column to remove unbound FITC. Solutions were made up fresh when required and filtered through a 0.22µm millipore filter.

10.3. PKH-2 labelling of AL DC

PKH-2 is an aliphatic fluorescent chromophore that becomes stably incorporated into membrane lipid bilayers (Horan *et al* 1990). PKH-2 has an emmision spectrum very similar to that of fluorescein and cells labelled with this dye can be detected using systems calibrated for detecting fluorescein activity.

DC were isolated from AL by centrifugation over 0.22 µm millipore filtered metrizamide (section 8.2). These cells were resuspended in RPMI containing 10% FCS and washed twice in this medium. A TNCC was made following resuspension in RPMI and the cells centrifuged (400rpm, 5 minutes) to form a loose pellet. These cells were then resuspended in 1ml of diluent to which 1ml of PKH-2 was added (appropriate concentration of PKH-2 previously determined from the concentration of DC). As a control, some of the cells were diluted in diluent alone. Following a 5 minute incubation at 20^0C, the cells were washed 3 times in RPMI containing 10% FCS. To determine the viability of the labelled cells, a small number were resuspended in PBS containing 1% propidium iodide (PI). PI uptake was determined by flow cytometry (section 11.4.) using the FACScan programme. By this method, DC viability was assessed to be greater than 95%.

11. Flow cytometric analysis of cell populations in body fluids (PB, SF and AL)

11.1. Preparation of single-cell suspensions prior to labelling with MAb

Heparinised whole blood was centrifuged (2500rpm, 20 mins) to obtain a buffy coat. To remove the red blood cells the buffy coat was added to warmed (37^0C) lysis buffer [9 parts NH_4Cl (0.83%w/v): 1 part TRIS (pH 7.6, 0.17M)], incubated for 2 minutes at this temperature, and then washed twice in cold PBS containing 2% BSA, 0.1% Az and 1i.u./ml heparin (cold buffer solution). For single-staining analysis, the cells were resuspended to a concentration of $2x10^6$/ml. For double-staining analysis, where the expression of different molecules on T lymphocyte subsets was evaluated and compared with T lymphocytes from SF, the final concentration of cells was determined by the concentration of cells in SF. In these studies, cells in PB and SF were resuspended to the same final cell concentration prior to labelling with Mab. This was done because the concentrations of cells in SF varied considerably and it was not possible to concentrate all samples to the same pre-determined concentration. Although this resulted in some inter-animal variation in cell concentrations used for this analysis, the intra-animal variation was minimised.

Following pelleting, the cells in SF were washed twice in cold buffer solution. Cells were resuspended in a sufficient volume of cold buffer solution for the number of tests required for analysis. There was also considerable variation in the concentration of cells in AL from different sheep. On these occasions, cells in AL were concentrated to the same level on each day of analysis.

11.2. Procedure for single staining of cell populations

Cells in 50µl of cold buffer solution (section 11.1.) were incubated with 50µl of Mab supernatant or diluted ascites (appropriate dilution predetermined) for 30 mins (4^0C). As a control, cells were incubated in normal mouse serum (1:500 in PBS/Az) or an isotype-matched Mab irrelevant to the study. Following two washes in cold buffer solution the cells were incubated in the appropiate FITC-conjugated antibody for 30 minutes (4^0C). Following two further washes in cold buffer solution the cells

were resuspended in 0.5% paraformaldehyde (pH 7.4) containing 0.1%Az and analysed within 24 hours.

11.3. Procedure for double staining of T lymphocytes

Cells were incubated in a mixture of 25µl of biotinylated anti-T lymphocyte Mab (first Mab) and 25 µl of Mab specific (different isotype or species-origin to the first Mab) for a second antigen (second Mab), for 30 mins (4^0C). As controls, the second Mab was replaced by an isotype-matched Mab irrelevant to the study, or normal mouse serum (1:500). Following two washes in cold buffer solution the cells were incubated in a 25µl of streptavidin-phycoerythrin (Amersham International plc, Amersham, UK) and 25µl FITC-conjugated antibody (species or isotype-specific depending upon the primary Mab; obtained from Binding Site Ltd, Birmingham) for 20 mins (4^0C). The optimal dilution of these secondary conjugated antibodies had been previously determined. Following one wash in cold buffer solution the cells were resuspended in 0.5% paraformaldehyde/Az.

11.4. Flow cytometric analysis of different cell populations in body fluids

All samples were analysed on a Becton Dickinson FACScan machine using Consort 30 (version 2.0) or Lysis (version 1.62) programmes. Single-cell suspensions obtained from body fluids contain a heterogenous population of cells. Individual populations can be identified on the basis of their characteristic forward (cell size) and side (heterogeneity of cell structure) light scatter profiles (Mason *et al* 1987). The scatter plots of PB, SF and AL are shown in Figure 3.1., with the location of the "live" gates indicated for each cell population that was analysed in this thesis. For lymphocytes in PB and AL, 5000 or 10000 cells were counted. For DC in AL, large mononuclear cells and lymphocytes in SF, it was not always possible to count this many cells. A minimum of 1000 cells were counted for each test performed on these cell populations.

Figure 3.1. Flow cytometric FSC/SSC profiles of cell populations in body fluids. Single-cell suspensions were obtained from PB (following ammonium chloride lysis of red blood cells)(a), SF (b) and AL (c) and analysed on a Becton Dickinson fluorescence-activated cell sorter. Box 1 in all three scatter plots was the "live" gate used for the analysis of lymphocyte populations and Box 2 was used for the analysis of larger mononuclear cells in SF (b) and DC in AL (c) respectively.

Figure 3.2. Flow cytometric analysis of background fluorescence of cell populations. The background fluorescence of lymphocytes in PB (A) and SF (B), larger mononuclear cells in SF (C) and DC in AL (D) are shown. For DC, a small number of cells with high autofluorescence were usually present. The percentage value for these cells obtained from the negative control was subtracted from the values for positive fluorescence for each Mab.

Figure 3.3. Background fluorescence of dual-stained lymphocytes in PB (a) and SF (b) evaluated by flow cytometry.

Briefly, single-cell populations in PB and SF were incubated in equal volumes of biotinylated normal mouse serum and Mab VPM53 (anti-Campylobacter). Following several washes the cells were incubated in equal volumes of phycoerythrin-labelled streptavidin and FITC-labelled anti-mouse Ig. Following one further wash and fixation the cells were analysed.

Figure 3.1

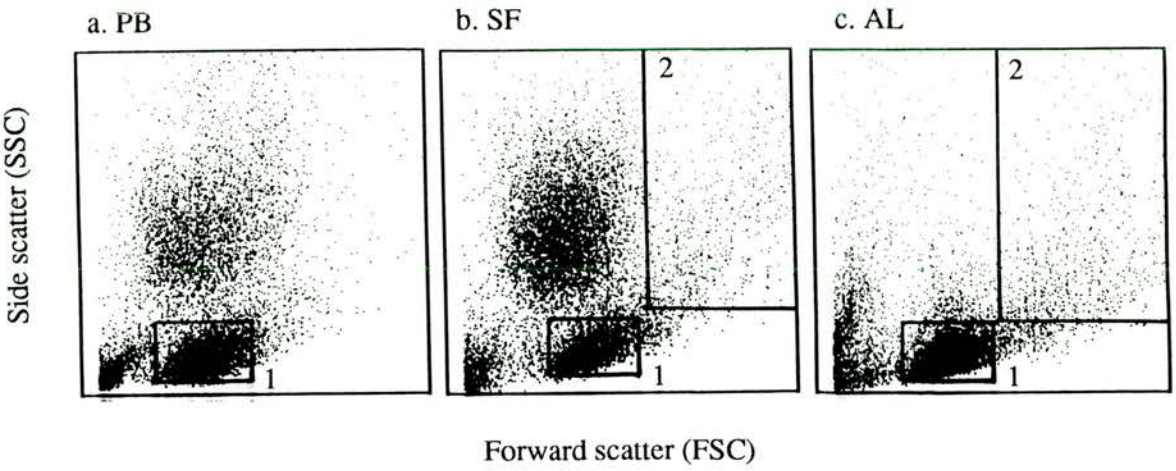


Figure 3.2

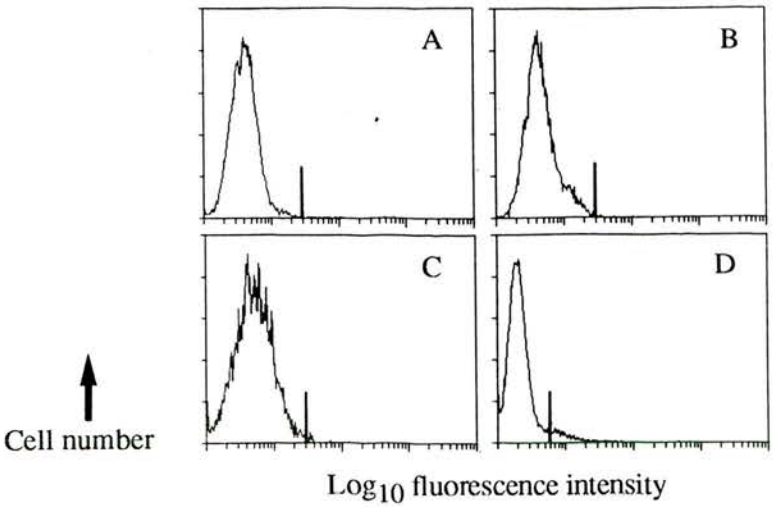
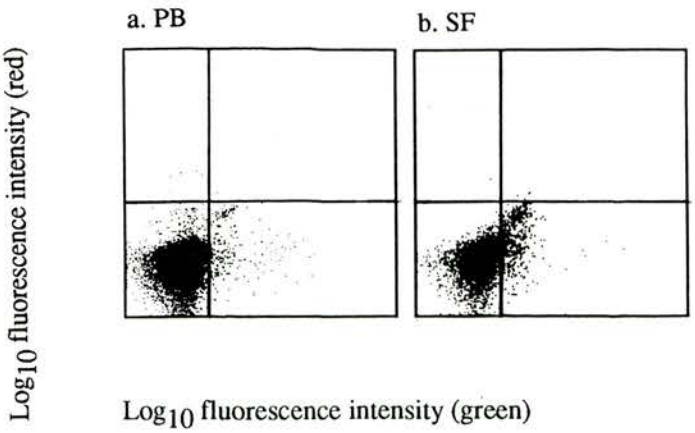


Figure 3.3



Determining the proportion of positively-fluorescent cells in each fluid sample was achieved by reference to the negative controls (Figure 3.2.). For single and double-staining of T lymphocytes in PB and AL, cursor placement defining positive fluorescence was straightforward. However, for SF the placement of cursors defining positive fluorescence was affected by the presence of cells with a high level of autofluorescence (Figure 3.3.). To prevent this interfering with the analysis, only those cells expressing high levels of fluorescence were regarded as positively stained, for the B and T lymphocyte antigens. This did not alter the results obtained with Mab to CD4, γδ TCR or DU2104+ B lymphocytes because of their uniformly high level of expression of the these antigens. The expression of CD8 and T19 was more variable and a small proportion of cells expressed low levels of these antigens. The above method of analysis, in some sheep, resulted in a small reduction in the percentage values obtained for cells expressing these antigens.

For each SF sample the percentage values obtained for the T and B lymphocytes were summated and the percentage value of each subset was recalculated as a percentage of the total lymphoid cells expressing these antigens. From these values were calculated the CD4:CD8, αβ:γδ and T:B lymphocyte ratios.

The identity of the cells with high autofluorescence was not established. Attempts to remove these cells by adherence by incubation of washed SF cells (in RPMI) on plastic plates (37^0C, 5% CO_2, 2 hours), and by centrifugation over Lymphoprep both failed. This would suggest that they were neither monocyte/macrophage nor dead cells. Further analysis to characterise these cells was not performed because they did not significantly interfere with the analysis of the expression of lymphocyte antigens.

To determine whether variation in cell concentration affected the proportion of cells positive for the lymphocyte antigens, different concentrations of lymphocytes from PB ($1x10^5$ to $1x10^7$/ml) were incubated in the same volume of Mabs used for single-staining analysis of these populations (Mab to CD4, CD8, γδ TCR and DU2104+ B lymphocytes). The results from this experiment showed that there was no

significant change in the percentages of cells positive for these antigens or in their intensity of expression over this range of cell concentrations (data not shown). This showed that variation in cell concentration in SF was unlikely to make a significant difference to the percentage values obtained for single-staining analysis with Mab to lymphocyte antigens.

Studies to determine the effects of hyaluronidase on the expression of all of the Mab described in this thesis showed that incubation (4^0C, 2 hours) of lymphocytes from PB in hyaluronidase at the concentration used in SF, had no effect upon the percentages of cells expressing these molecules or the level of intensity of their expression (data not shown). The 2 hour incubation period was chosen because this was the longest duration of time that SF was incubating with hyaluronidase (at 4^0C)(Most sheep used in this study were kept at a distance of 20 minutes by car from the Department of Veterinary Pathology, R(D)SVS, Edinburgh).

12. Measurement of antibody in body fluids

12.1. Measurement of anti-ovalbumin IgG antibody in serum, SF and AL by ELISA.

96 well flat-bottomed ELISA plates (Dynatech, Billinghurst, Sussex) were incubated overnight (4^0C) with 10μg/ml OVA in PBS containing 0.01% azide. Following 3 washes in PBS (pH 7.4) containing 0.1% Tween 20, doubling dilutions of body fluid (diluted in PBS containing 2% BSA) were added in triplicate to the wells and incubated for 1 hour at room temperature. The plates were washed three times in PBS/Tween and then incubated for 30 mins in alkaline phosphatase-labelled anti-sheep IgG (diluted 1:1000 in BSA solution). After a further 3 washes in PBS/Tween, enzyme substrate (0.1M glycine, 0.05M sodium hydroxide, 0.0005M magnesium chloride, 0.0005M zinc chloride and 10mg Sigma powder 104 per 10mls distilled water) was added and the plates incubated for 20 mins at 37^0C. The plates were read at 405nm in a Titertek plate reader. The highest dilution of body fluid that gave an

optical density value of double that obtained with BSA alone was taken to be the antibody titre for that sample.

12.2. Ouchterlony tests for determining antibody:antigen equivalence.

1% agarose solution was made by dissolving agarose in warmed PBS (pH 7.4) containing 0.01%Az. This was then poured onto glass plates covered with agarose gel support medium (Gelbond film; FMC Bioproducts, Rockland, ME04841, USA) and allowed to set in a humidity chamber at room temperature. 4mm holes were punched out of the gel with the aid of a template and doubling dilutions of OVA solutions (in PBS/Az) were pipetted into the outer wells. Serum or SF containing OVA-specific antibody was placed in the central well. Gels were then incubated in a humidity chamber at room temperature until precipitin lines had formed. The gel was extensively washed in water baths and then stained for several minutes in 0.25% Coommassie blue solution (45% methanol, 10% glacial acetic acid). After washing in destain solution (10% methanol, 5% glacial acetic acid), gels were placed between pieces of absorbent paper and placed under a heavy weight for several days. The equivalence point was the concentration of antigen at which the most clearly delineated precipitin line had formed.

13. T lymphocte proliferation assays

13.1. T lymphocyte proliferation assays with PBMC

T lymphocyte proliferation assays were performed with PBMC and SF mononuclear cells. PBMC were isolated from whole defibrinated blood by centrifugation over Lymphoprep (20 mins, 3000rpm). Following three washes in RPMI containing 2% FCS the cells were counted, their viability assessed (1% Trypan blue exclusion) and finally resuspended to a concentration of $5x10^5$/ml in RPMI containing 10% FCS, 100i.u./ml penicillin, 100i.u./ml streptomycin, 2mM L-glutamine and $5x10^{-5}$M 2-mercaptoethanol. $5x10^4$ cells in 100µl medium were added to each well in 96 well round-bottomed plates (Dynatech, Billinghurst, Sussex). Dilutions of

antigen (OVA or HSA in RPMI and filtered through a 0.22μm millipore filter) were added to wells (100μl/well) in triplicate to give final antigen dilutions ranging from 100μg/ml to 0.38μg/ml. Concanavalin A (Con A)(10μg/ml) and medium alone were used as positive and negative controls respectively. Following 5 days incubation (37^0C, 5% CO_2) the cells were pulsed with tritiated methyl thymidine (^{3}H-TdR), incubated a further 5 hours and then harvested on a Tomtec Harvester 96. ^{3}H-TdR incorporation was measured on a 1450 Microbeta plus, liquid scintillation counter (Wallac). The results were analysed by the Mann Whitney non-parametric statistical tests.

13.2. T lymphocyte proliferation assays with SF cells

SF was aspirated under aseptic conditions and mixed with RPMI containing 10i.u. heparin/ml. The joint was then flushed with 2mls of RPMI and re-aspirated to increase cell recovery. Following centrifugation (1000rpm, 5 mins) the cells were resuspended in RPMI containing 2% FCS, washed once in this medium and centrifuged over Lymphoprep (section 8.1.). The remainder of the protocol was identical to that for PBMC.

14. Surgical procedures

14.1 Induction and maintenance of general anaesthesia

General anaesthesia was induced by the intravenous injection of 4mg/kg alphaxolone and 1.3mg/kg alphadolone (Saffan; Pitman-Moore Ltd, Crewe, Cheshire). Following intubation, gaseous anaesthesia was maintained with a balanced mixture of halothane (M&B, Rhone Merieux Ltd, Harlow), oxygen and nitrous oxide delivered in a rebreathing circuit. The procedures described in sections 14.2. and 14.3. were performed on sheep under general anaesthesia.

14.2. Biopsy of synovial lining of the RC and TT joints

The anaesthetised sheep was placed in lateral recumbency and the hair clipped from the area of the limb in the immediate vicinity of the RC joint. Following preparation of the skin the surgical field was draped. A vertical skin incision extending approximately 4 cms was made in the midline, centred on the RC joint. The underlying subcutaneous tissue was dissected down to the synovial sheath surrounding the tendon of the *extensor carpi radialis* muscle. Deviation of the tendon allowed access to joint capsule and a portion of the synovial lining was removed together with the joint capsule on the inner aspect of this tendon. Wound closure was achieved by apposition of deep and superficial fascial layers with a continuous suture of 2-0 polyglactin (Vicryl; Ethicon Ltd, Edinburgh). Simple interrupted sutures with the same suture material were placed in the skin to avoid the necessity for suture removal. A lightly padded dressing was applied to the surgical area to protect the wound for the first week after surgery.

For the TT joint, a 4cm skin incision was made on the anteromedial aspect of the joint to expose the tendon of the *cranial tibial* muscle. The synovial lining of this joint was biopsied immediately adjacent to this tendon and wound closure was as described for the RC joint. Post-operative prophylactic antibiosis was maintained for three days with intramuscular injections of 1500i.u./kg procaine penicillin G and 12mg/kg dihydrostreptomycin (Penstrep; C-Vet Ltd, Bury St. Edmunds, Suffolk).

14.3. Popliteal lymphadenectomy and cannulation of the popliteal pseudoafferent lymphatic vessel

Bilateral popliteal lymphadenectomy was performed to allow the formation of pseudoafferent lymphatic vessels. The afferent and efferent lymphatic vessels previously entering and leaving the lymph node respectively, anastomose within several weeks of lymphadenectomy (Hopkins *et al* 1985). With the sheep in lateral recumbency the skin caudal to the stifle joint was clipped and prepared for surgery. The popliteal lymph node was removed through a small incision between the caudal

edge of the *biceps femoris* muscle and the *semitendinosus* muscle. Care was taken to keep soft tissue dissection to a minimum and to confine to it to tissue below a transverse plane through the stifle joint. This was done in an effort to keep subsequent scar tissue formation to a minimum.

A minimum period of 6 weeks was then allowed to elapse before attempting to cannulate the pseudoafferent lymphatic vessel. Prior to surgery, 0.5 ml of sterile 2% Evans blue was injected intradermally proximal to the TT joint. This subsequently allowed identification of the lymphatic vessels. The surgical approach to the pseudoafferent lymphatic was similar to that for lymphadenectomy except that soft tissue dissection was confined to the tissues proximal to the scar tissue from the previous surgery. Following their identification the lymphatic vessels were isolated and ligated proximally. A metal probe was passed through the fascial plane between the *biceps femoris* and *semitendinosus* muscles and out through the skin immediately caudal to the greater trochanter of the femur. A heparinised plastic cannula (External diameter 0.98mm)(Portex, UK) was drawn back through this tunnel, inserted into the pseudoafferent vessel and sutured in place with several ligatures of 2-0 polyglactin. Following apposition of the superficial and deep fascial planes with a continuous suture of 2-0 polyglactin, simple interupted sutures were placed in the skin. Lymph was collected in a sterilised 250 ml plastic bottle containing 250 i.u. heparin and 2500 i.u. of procaine penicillin G and dihydrostreptamycin. When the volume of lymph collected was small, a 15ml sterile plastic tube was used, with an appropriate reduction in the quantities of heparin and antibiotic. These vessels were attached to the animals flank immediately cranial to the limb, by means of a plastic plate sutured to the skin. Perioperative antibiosis was provided in the same fashion as described in section 14.2. Sheep were kept in small, strawed pens and fed a diet of hay, commercial meal and water *ad lib*.

15. Statistical analysis

Non-parametric statistical tests were performed in the analysis of data in this thesis. These included the Mann Whitney U-test, Kruskal-Wallis test (for the analysis of variance (ANOVA)] and Wilcoxon Signed Rank tests where data was paired.

CHAPTER 3

Phenotypic characterisation of synovial tissues from different stages of development

Introduction

During development the synovial lining arises from the mesenchyme in the articular interzone (O'Rahilly and Gardner 1978). The developmental changes of synovium have been studied in several species including humans (Anderson 1964), rats (Izumi *et al* 1990) and mice (Takabataka *et al* 1991). With the invasion of blood vessels into the synovial mesenchyme and the appearance of the articular cleft that enlarges to form the joint cavity, cells appear in the SI and SLC layers with the ultrastructural, cytochemical and immunocytochemical features of macrophages (Anderson 1964, Izumi *et al* 1990, Takabataka *et al* 1991), together with small numbers of other white blood cells and mast cells (Anderson 1964, Krey *et al* 1971).

Very few studies have been performed to characterise synovial tissues from the joints of normal sheep at any stage of development. Cutlip and Cheville (1973) examined synovia from the stifle joints of 3 month old lambs and confirmed the presence of type A and type B cells in the SLC layer and identified the 3 major types of synovial surface; areolar, fibrous and adipose. Immunocytochemical studies of synovial tissues have rarely been performed although Harkiss *et al* (1991) have characterised the phenotype of LMN cells and the B and T lymphocyte subsets in SF from different joints of normal adult sheep by flow cytometry.

The aim of the work described in this Chapter was to characterise the basic histological, immunocytochemical and cytochemical features of the synovial lining from normal sheep at different stages of development from the foetus to the adult. Additionally, to evaluate further whether there were age-related changes in synovial tissues, LMN cells in SF were evaluated for their expression of a limited range of cell surface molecules by flow cytometry.

Outline of Methods

Groups of lambs were euthanased at defined time intervals after birth (under 1 week, 3 to 4 weeks and 3 to 4 months of age) and synovial tissues were obtained from one or both RC joints. Synovial tissues from foetal lambs and adult sheep were also examined. The age of these foetal lambs was estimated from the formula in Chapter 2, section 2.1.. Portions of synovium were processed for immunocytochemistry and routine histological examination (stained with H+E). Immunocytochemistry was performed on frozen sections with a panel of Mab to MHC class II (DRα and DQα chains), CD14, FcγRII, CD11c (quantitated according to method in Chapter 2, section 6.4.2.), and B (DU2104+) and T lymphocyte subsets (CD4+, CD8+ and $\gamma\delta$). NSE staining of frozen sections was also performed.

SF was aspirated from some of these joints and evaluated by TNCC and differential cell counts from cytospin preparations. In a smaller number of cases the phenotypic expression of MHC class II (DRα and DQα chains) and CD14 by LMN cells was evaluated by flow cytometry.

Results

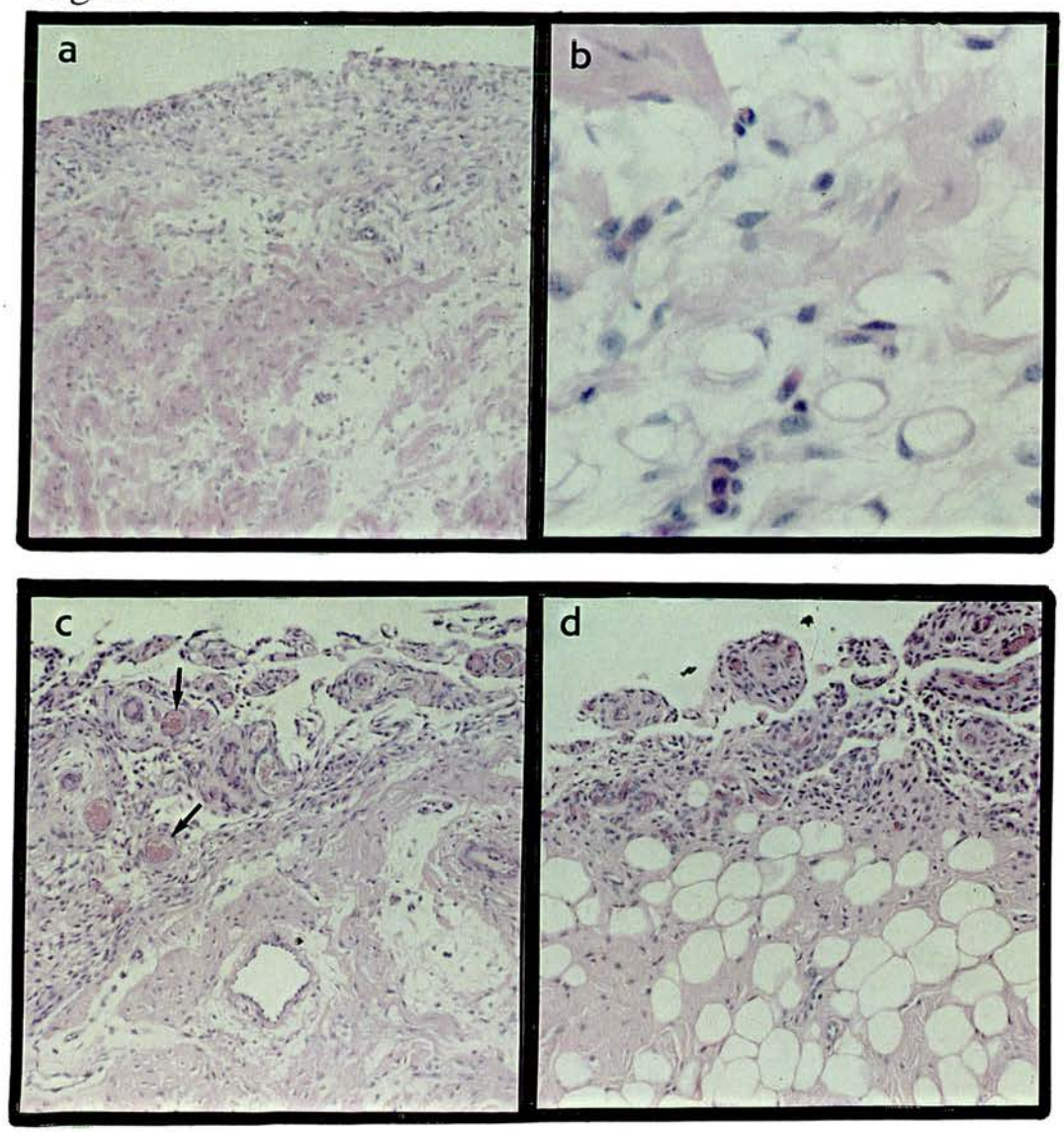
1. Histological descriptions of synovium

1.1. Foetal lambs (n=10)

The age of 6 of 10 foetuses was estimated from the date of service to be approximately 120 to 132 days (average gestation length in sheep is 150 days). The remaining tissues were from foetuses of 80 to 100 days of age. The appearance of the synovium from the younger age group was slightly different to that from the older group in that a distinct SLC layer was not clearly distinguishable (Figure 1). This was due to the highly cellular nature of the SI and a lack of clear differentiation of the mesenchymal cells to give a definable characteristic (ie areolar, fibrous or adipose) to the synovium. Large numbers of cells had large,

Figure 1. The histology of synovium from RC joints of lambs of different ages. (a) and (b)(original magnification x156 and x624 respectively), synovium from a 90 day foetus. (c), synovium from a 1 week old and (d) 3 month old sheep (original magnification, x156). The arrow in Figure (c) shows a blood vessel with partial occlusion of its lumen by eosinophilic proteinaceous debris.

Figure 1



rounded nuclei with varying quantities of cytoplasm that often formed processes of varying length. Developing adipocytes and large active fibroblasts interspersed with irregular bundles of immature collagen fibres were present throughout the SI (Figure 1). Only occasional cells with a lymphoid appearance were observed. Moderate numbers of small blood vessels were present throughout the SI.

Later in gestation, adipocytes were more prominent in the SI and a SLC layer was more clearly identifiable compared to earlier in gestation. Generally the SLC layer was 1 to 2 cells thick but this was increased focally to 6 to 8 cells. Large numbers of capillaries were present immediately beneath and amongst the SLC and occasional lymphoid cells were observed in the SI. Villiform folding was present in tissues from several foetuses which had not been observed in synovia from earlier in gestation.

1.2. Lambs under 1 week of age (n=10)

Synovium from these lambs closely resembled that from foetuses from late in gestation. In all tissues the SLC layer was 1 to 2 cells thick and some degree of villiform folding was present (Figure 1). Occasional scattered lymphoid cells were present in the majority of sections examined (13 of 16 tissues). Small numbers of neutrophils were observed immediately beneath the SLC in 3 of 16 tissues and many of the most superficial blood vessels were congested and their lumens were partially occluded by homogenous eosinophilic proteinaceous debris (Figure 1).

1.3. Lambs 3 to 4 weeks of age (n=12)

The synovium appeared similar to that from the previous age group except that adipose tissue appeared more prominent. Occasional scattered lymphocytes were observed in most tissues and small numbers of neutrophils were present in 5 of 18 tissues. No blood vessels with eosinophilic proteinaceous debris in their lumena were observed.

1.4. Lambs 3 to 4 months of age (n=10)

The synovium appeared very similar to that from the previous age group except that no neutrophils were observed in any of the sections (Figure 1).

1.5. Adult sheep of minimum age 4 years (n=15)

A full description of the features of synovium from these sheep is given in Chapter 4, section 3.1. and 4.1.. Briefly, mild SLC hyperplasia was present in 7 of 15 sheep and many tissues showed replacement of adipose tissue with fibrous tissue. Small to moderate numbers of scattered lymphoid cells were present in 3 of 15 tissues.

2. Immunohistological features of synovium

2.1. Foetal synovium (n=5)

Synovium from 5 foetuses at 120 to 132 days of gestation was examined. Very small numbers of MHC class II+ SLC were present in all tissues (Figure 2). Although frequently present as solitary cells, occasional small aggregates of positively-stained cells were observed. Small numbers of positively-stained cells were scattered throughout the SI in all tissues. These cells were usually quite large with abundant cytoplasm and some possessed short cytoplasmic processes (Figure 2), although occasional cells were smaller and more spindle-shaped. Blood vessels with MHC class II+ endothelial linings were rarely observed. The majority of SLC in all tissues were CD14+ and FcγRII+ (Figure 3). Small numbers of cells positively-stained for these antigens were scattered throughout the SI. In all tissues the numbers of these cells exceeded the number of MHC class II+ cells. In 2 of 6 tissues, occasional CD4+, CD8+ and γδ T lymphocytes were present. However, no DU2104+ B cells and very few NSE+ cells were observed.

Figure 2. MHC class II expression by synovium from the RC joints of sheep of different ages (Immunoperoxidase technique).

(a) and (b)(original magnification x156 and x624 respectively), synovium from a 120 day foetus. The arrow in (a) shows a cluster of positively-stained SLC and that in (b) shows a large positively-stained cell in the SI. (c), synovium from a 1 week old sheep. Moderate numbers of positively-stained cells are present in the SI. (d), synovium from an adult sheep. The small arrow shows a blood vessel with positively-stained endothelium and the open arrow shows a positively-stained cell located perivascularly.

Figure 2

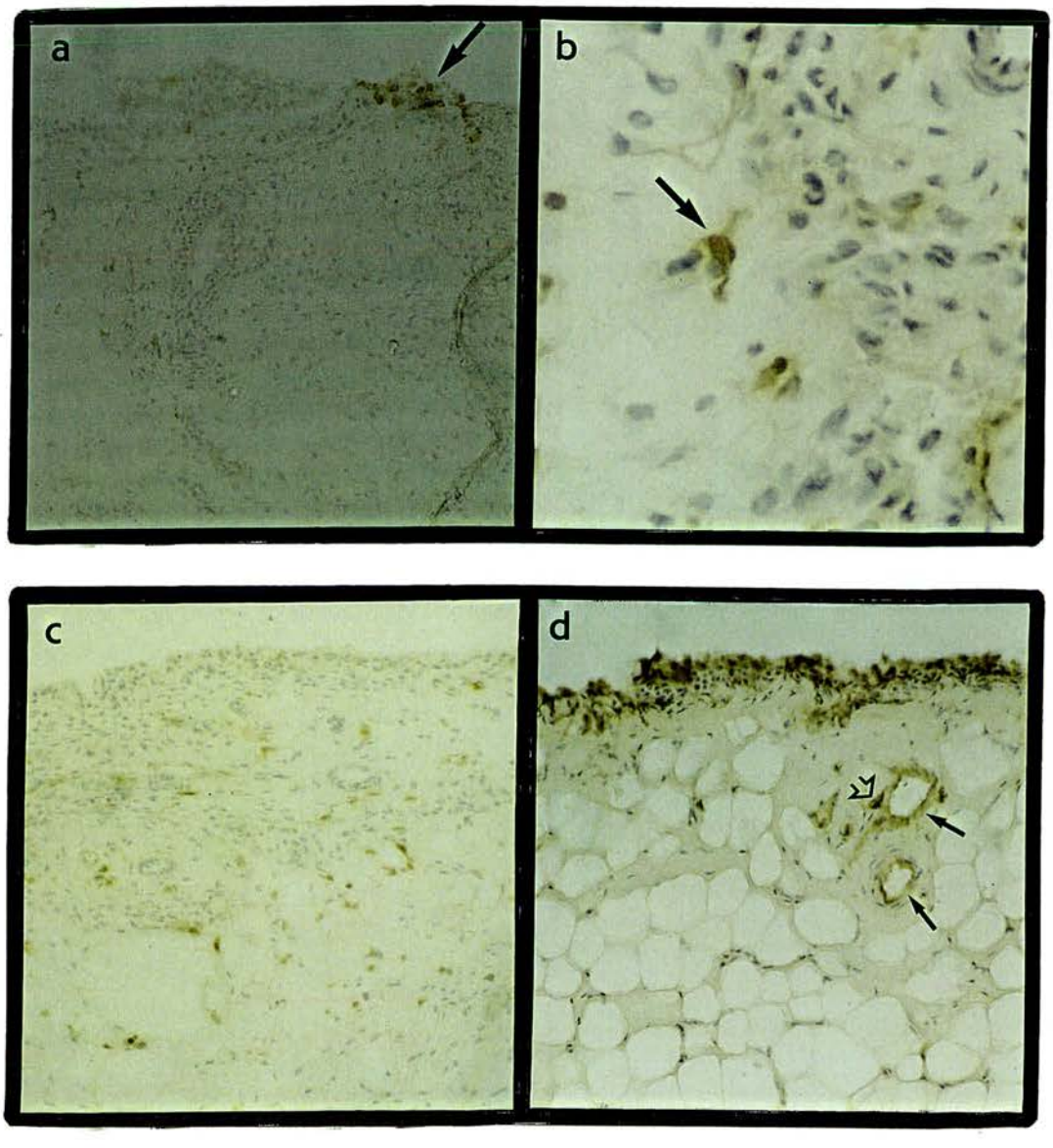
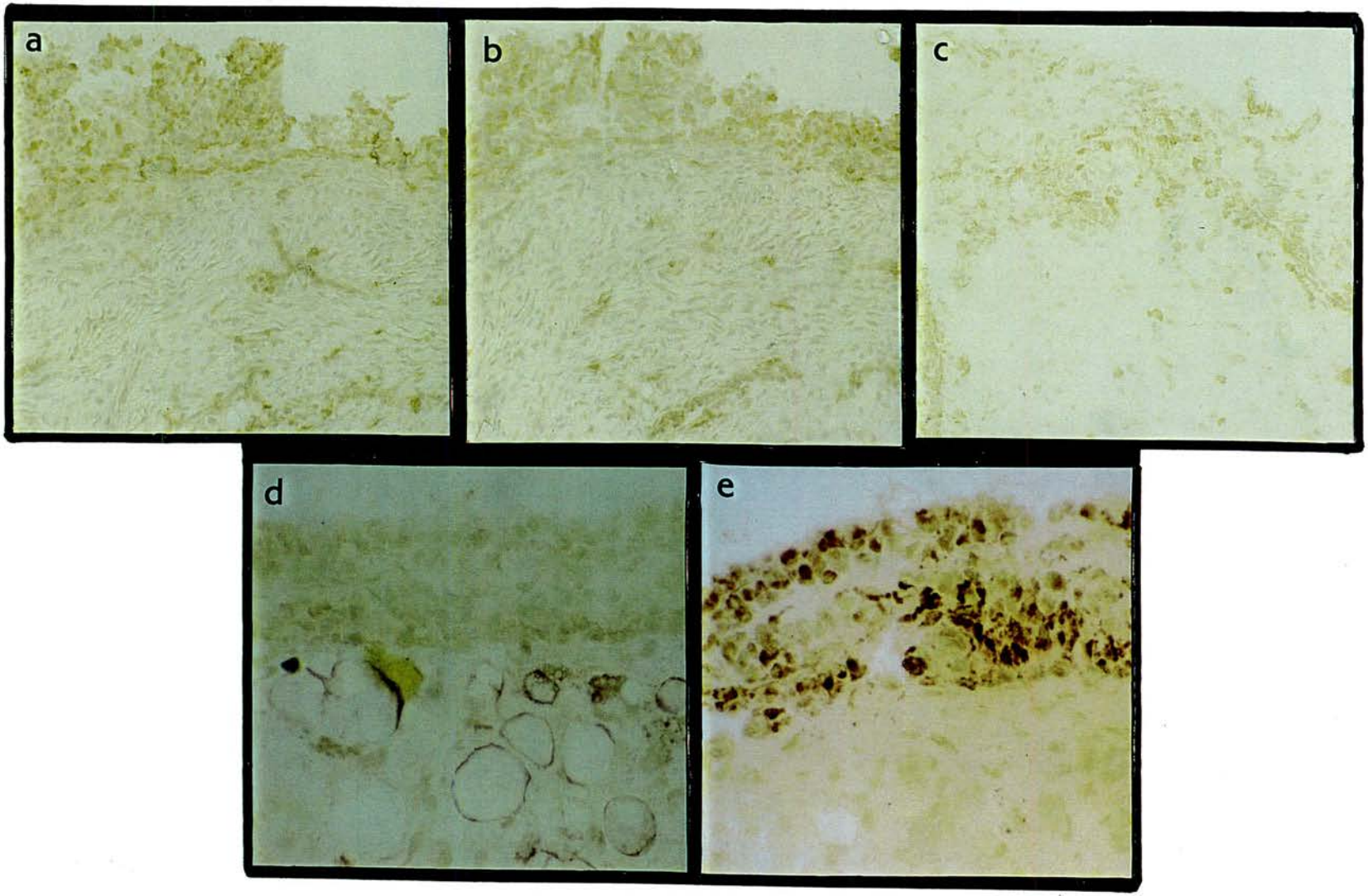


Figure 3. Immunohistological and histochemical characteristics of synovium.

(a), (b) and (c) are synovium from a 120 day foetus labelled with Mab to CD14, FcγRII and CD11c respectively (Immunoperoxidase technique, original magnification x156). (d) and (e) are synovium from a 120 day foetus and a 1 month old sheep respectively, stained for NSE activity (original magnification x390).

Figure 3



2.2. Synovium from lambs under 1 week of age (n=5)

The expression of MHC class II (Figure 2), CD14 and FcγRII by SLC and cells in the SI closely resembled that observed in foetal tissues. In addition, most sections had some weakly MHC class II+ endothelial cells. The number of CD11c+ cells appeared quite variable with moderate numbers of positively-stained SLC and cells in the SI in some sections (Figure 3) whilst other sections had few positively-stained cells in either layer. Single CD4+, CD8+ and γδ T lymphocytes were observed in 2 of 5 tissues. However, no DU2104+ B cells were observed. The pattern of NSE staining was the same as described for tissues from the foetus.

2.3. Synovium from lambs 3 to 4 weeks of age (n=4)

The expression of CD14, CD11c and FcγRII by the SLC was very similar to the previous age group. However, the majority of these cells expressed MHC class II. Also, the numbers of CD14+, CD11c+ and FcγRII+ cells in the SI appeared similar to the previous age group and these exceeded the numbers of MHC class II-expressing cells in the SI. Small to moderate numbers of smaller blood vessels had MHC class II+ endothelial linings. Occasional T lymphocytes of all three subsets were observed in 2 of 4 tissues but no DU2104+ B cells were observed. The number of NSE+ cells was considerably increased compared to the previous age group and the majority of these cells were located in the SLC layer. These cells were distributed unevenly throughout the SLC layer with some areas devoid of positively-stained cells whereas in other areas the majority of cells were positively-stained (Figure 3).

2.4. Synovium from lambs 3 to 4 months of age (n=4)

Synovium from these lambs had a similar appearance to those from the previous age group in terms of numbers and distribution of cells positively-stained for all Mab and NSE. The above results are summarised in Table 1.

Table 1. Summary of immunohistological and NSE staining characteristics of synovium from sheep of different ages.

Age[a]		MHC class II[b]	CD14	FcγRII	CD11c	NSE
Foetus (5/5)	SLC	+ (- / +)	+++	+++	ND	- (-/+)
	SI	+	++	++		+
<1 week (4/4)	SLC	+ (- / +)	+++	+++	++ (+/++)	+ (-/+)
	SI	+ (+ / ++)	++	++	+ (-/+)	+
3-4 weeks (4/4)	SLC	+++	+++	+++	++ (+/+++)	++ (+/+++)
	SI	+ (+ / ++)	++	++	+ (+/++)	+
3-4 months (4/4)	SLC	+++	+++	+++	++ (+/+++)	++ (+/+++)
	SI	+ (+ / ++)	++	++	+ (+/++)	+

Key

- absent

+ occasional positively-stained cells

++ moderate numbers of positively-stained cells

+++ majority of cells positively-stained

[a] The values in parentheses show the number of joints (first figure) from the number of sheep (second figure) examined.

[b] There was no observable difference in the expression of MHC class II DR or DQ antigens.

The values represent the commonest finding for the age group. Values in parentheses indicate the range of observations when inter-individual variation was observed.

2.5. Adult sheep of minimum age 4 years (n=10)

A detailed description of the immunohistological characteristics of synovia, using a more restricted range of Mab, from these sheep is given in Chapter 4 section 3.1. and 4.1.. There appeared to be little difference in the number or distribution of MHC class II+ cells in the synovia of these sheep compared to lambs at 3 to 4 months of age (Figure 2). However, in two tissues moderate numbers of MHC class II+ cell were present in the SI. Small numbers of T lymphocytes of all 3 subsets were present in 9 of 10 tissues. Generally the number of CD4+ T lymphocytes exceeded the number of CD8+ or γδ T cells and only very occasional DU2104+ B cells were observed. NSE staining was mainly confined to cells in the SLC layer, with occasional positively-stained cells in the SI.

3. SF analysis

3.1. TNCC and differential cell counts

The SF TNCC and differential cell counts for SF from the RC joints of lambs of different ages are shown in Table 2. Because of the paucity of SF in the RC joints of foetal lambs, SF from both RC joints were pooled for analysis. ANOVA (Kruskal-Wallis) was performed initially to assess whether there were significant differences in TNCC of SF from different age groups. Further statistical analysis was performed using the Mann Whitney U-test.

ANOVA of the TNCC confirmed that there were significant differences between the lambs of different ages ($p<0.001$). The SF TNCC from lambs under 1 week of age was significantly higher than from all other age groups ($p<0.001$). Neutrophils formed a higher percentage of the cells in SF from lambs under 1 week and under 1 month of age than at the other time points. Although not quantitated, larger numbers of cells undergoing division were observed in SF from foetal and lambs under 1 week of age compared to the older lambs.

Table 2. Synovial fluid differential cell counts from the RC joints of sheep of different ages. The values shown are the mean ± s.d. and the range is shown in parentheses.

Age[a]	TNCC ($x10^5$/ml)	Neutrophils %	Lymphocytes %	Synoviocytes %	Macrophages %
Foetal (12/6)	2.2 ± 1 (0.8-3.6)	0	4.7 ± 1.2 (2.5-6)	87 ± 1.8 (85-90)	8 ± 0.9 (7-9.5)
< 1 week (12/7)	*18 ± 20 (4-65)	25 ± 31 (0-82)	6.8 ± 4.4 (0.5-15)	43 ± 25 (2-69)	25 ± 10 (11-41)
3-4 weeks (16/10)	2.8 ± 2.3 (0.8-8)	17 ± 30 (0-90)	11 ± 9 (1.5-40)	65 ± 32 (0-93)	6.7 ± 6 (0-17)
3-4 months (15/9)	2.2 ± 1.3 (0.7-4)	0.2 ± 0.4 (0-1)	4.8 ± 2.3 (0.5-9)	91 ± 4.8 (84-97)	4.3 ± 3.5 (0-10)

[a] The values in parentheses show the number of SF analysed (first figure) and the number of sheep from which these were obtained (second figure).

*The TNCC of SF from lambs of under 1 week of age was significantly higher than all other age group ($p<0.001$ for all comparisons).

3.2. Flow cytometric analysis of LMN cells in SF

The expression of MHC class II (DRα and DQα) and CD14 by SF LMN cells from sheep of different ages was determined by flow cytometry. SF was obtained from both RC joints of 6 sheep at under 1 week, 3 to 4 weeks and 3 to 4 months of age. The "live" gate used to analyse this population was shown in Chapter 2, Figure 3.1.. Care was taken to ensure that the same "live" gate was used for the analysis of each sample. For statistical analysis, the percentage values for MHC class II DRα and DQα antigen expression and the mean fluorescence intensity (MFI) values were initially compared by ANOVA (Kruskal-Wallis) and further comparisons were made using a Mann Whitney U-test.

The proportion of LMN cells expressing MHC class II antigens and the intensity of expression of these antigens, assessed from the MFI values, significantly changed with age ($p<0.001$)(Figure 4, 5.1 and 5.2). The percentage of these cells expressing MHC class II antigens in lambs under 1 week of age was low. At one month of age, the proportion of LMN cells expressing the DRα chain was significantly higher than the values obtained at 1 week ($p<0.0001$) and there was a further significant increase at 4 months of age ($p<0.014$). Although the proportion of LMN cells expressing DQα was significantly higher at 1 month compared to 1 week ($p<0.0001$), the p value obtained on comparison with LMN cells from lambs at 4 months of age did not reach significance ($p=0.056$). Although there was no significant difference in the intensity of MHC class II expression by SF LMN cells from lambs at one month compared to one week of age, the MFI values for DRα and DQα expression at 4 months were significantly higher than those obtained at 4 weeks ($p<0.0001$ for both antigens). The percentage of CD14+ LMN cells in SF always exceeded or equalled that obtained for either of the MHC class II antigens.

Figure 4. Expression of MHC class II and CD14 by SF LMN evaluated by flow cytometry.

Following labelling with Mab to MHC class II DRα and DQα chains (A) and CD14 (B), SF LMN cells were analysed by flow cytometry using the "live" gate shown in Chapter 2, Figure 3.1b. 2000 cells were analysed for each Mab in each SF sample. As a control, cells were incubated with the isotype-matched Mab VPM53 (anti-Campylobacter).

Figure 4

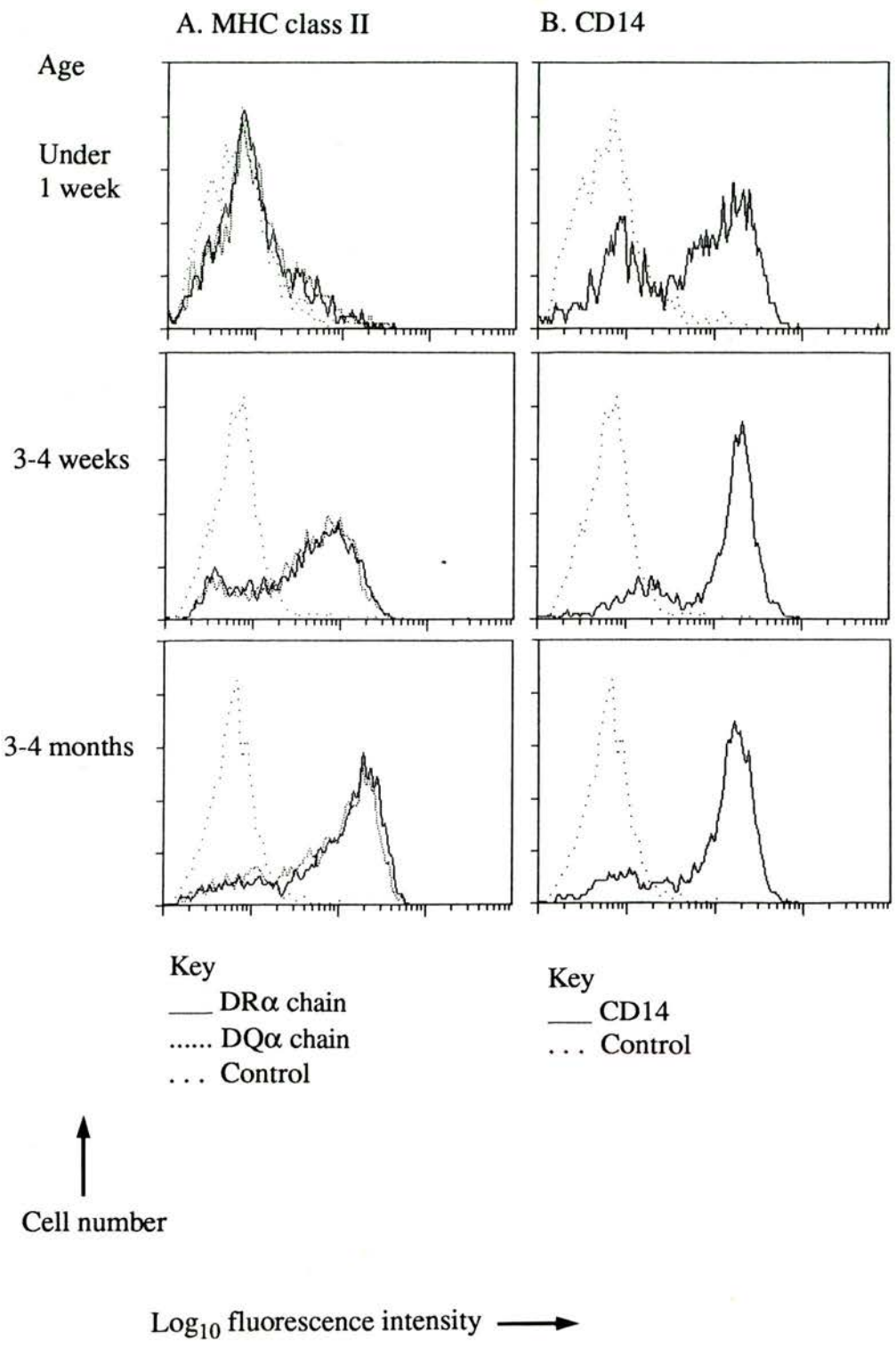

A. MHC class II
B. CD14
Age
Under 1 week
3-4 weeks
3-4 months
Key
___ DRα chain
..... DQα chain
. . . Control
Key
___ CD14
. . . Control
Cell number
Log10 fluorescence intensity

Figure 5.1 and 5.2. Expression of MHC class II (DR and DQ) and CD14 antigens by LMN cells in SF from sheep of different ages.

Figure 5.1 shows the percentage of LMN cells in SF expressing MHC class II (DR and DQ) and CD14 antigens from sheep at 1 week, 4 weeks and 4 months of age, determined by flow cytometry (*$p<0.0001$ compared to values at 1 week, †$p<0.014$ compared to value at 1 month)

Figure 5.2 shows the intensity of expression of MHC class II DR and DQ antigens by SF LMN cells from sheep of different ages, determined by the mean fluorescence intensity values obtained by flow cytometry. (*$p<0.0001$ compared to values at 1 month).

Figure 5.1

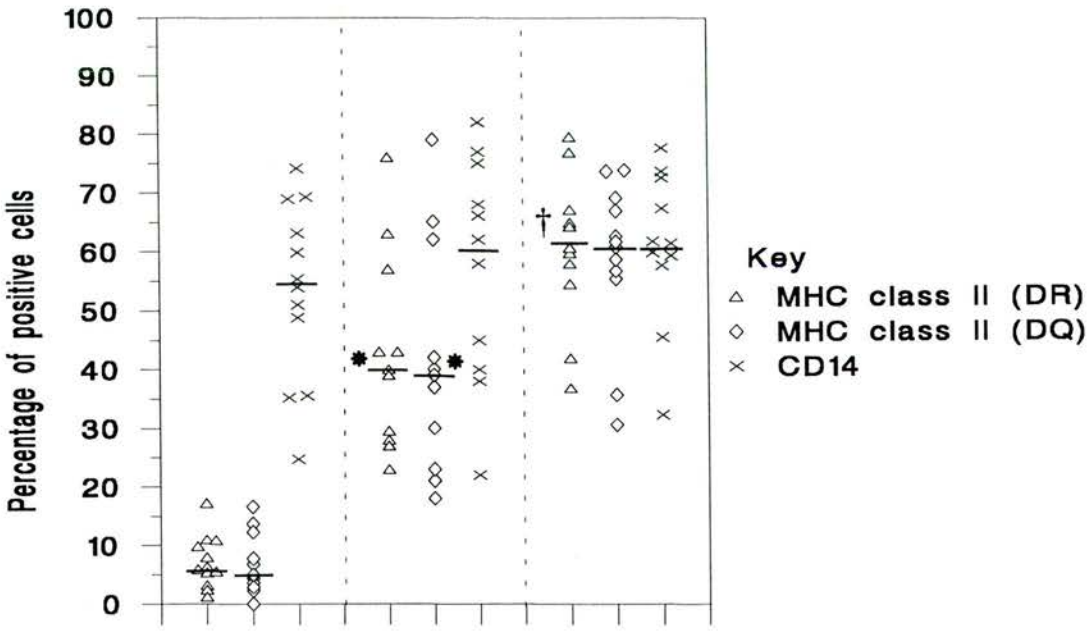
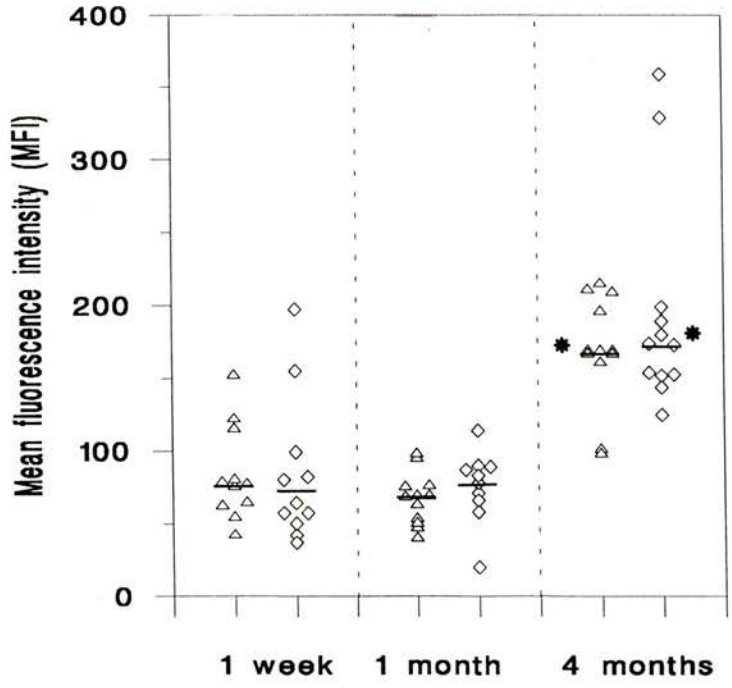


Figure 5.2



Summary of findings

1. The histological appearance of synovium from the RC joint of sheep changes significantly during the period of development from late in foetal life to adulthood.

2. Immunohistological examination of synovium revealed that cells expressing CD14, FcγRII, CD11c and MHC class II were present in all ages of sheep examined. Additionally, small numbers of T lymphocytes, but not B lymphocytes recognised by Mab DU2104, were identified in tissues from all ages of sheep.

3. Small numbers of cells in the SLC layer expressed MHC class II in synovia from foetuses and newborn sheep. Their numbers were markedy increased in synovia from older sheep. Additionally, the numbers of LMN cells in SF expressing MHC class II and its intensity of expression, evaluated by flow cytometry, increased significantly in sheep from the time of birth to 4 months of age.

4. Significant inflammatory changes were present in SF and synovia from sheep during the first month of neonatal life. However, the cause of these changes was not established.

Discussion

The basic histological appearance of synovium from foetal lambs was similar to descriptions of foetal synovium from humans, rats and mice (Krey *et al* 1971, Izumi *et al* 1990, Takabataka *et al* 1991). Also, the appearance of synovium from the RC joints of 3 to 4 month old lambs was similar to that previously described for synovium from the stifle joints of lambs of a similar age (Cheville and Cutlip 1973).

The immunohistological studies of synovium and flow cytometric analysis of LMN cells in SF showed that there were age-related changes in the expression of MHC class II. Whether similar changes occur in joints other than the RC joint was not established. Similar to previous studies of synovia from the foetal rats, only small numbers of cells expressing MHC class II were present in foetal sheep synovium. The identity of these cells in the SI was not established (with the

exception of occasional endothelial cells) because double-staining procedures were not performed. However, the vast majority of positively-stained cells appeared to be larger than lymphoid cells, which were present in very low numbers, and therefore these cells were likely to be macrophages, fibroblasts, mesenchymal cells or DC. At 3 to 4 weeks of age there was a marked increase in the numbers of cells expressing MHC class II in the SLC layer. However, it is unclear whether these positively-stained cells were resident at the time of birth and had been induced to express MHC class II or whether they were recently-arrived monocyte-derived cells which expressed MHC class II following entry into the synovium.

On the basis of studies to determine the incorporation of giant granule-containing cells into the SLC layer of synovium in beige mice (Edwards 1982), Henderson and Edwards (1987) have calculated that the rate of turnover of SLC in normal mouse synovium is of the order of 20 weeks for type A cells and approximately 1 year for type B cells. These figures would suggest that most of the increase was due to resident cells being activated to express these antigens. However, the rate of turnover of SLC is likely to be higher in the synovium of young growing animals and the inflammatory changes that were evident in joint tissues during the first month after birth would also increase the turnover of these cells, either by recruitment of monocytes from the peripheral circulation or by increased local cell division (Henderson *et al* 1982).

The increased numbers of SLC expressing MHC class II was paralleled by an increase in the percentage of LMN cells in SF expressing these antigens. The identity of these MHC class II-expressing cells in SF was not established because double-staining procedures were not performed. However, the majority of LMN cells within the "live" gate used for the analysis of these cells were monocyte-derived and monocytes in sheep express MHC class II DR and DQ antigens (Dr J. Hopkins, personal communication). The CD14 - population of cells may have been monocyte-derived cells that had down-regulated their expression of this molecule

or alternatively they may have been fibroblast-like cells or possibly DC which express very low levels of CD14 (Gupta 1994). It is not known whether there are age-related changes in the expression of MHC class II by monocytes in sheep similar to humans where fewer cord monocytes express MHC class II antigens compared to the adult (Edwards *et al* 1985). However, this would help to explain the low numbers of MHC class II-expressing SLC in synovia from foetuses, many of which were clearly monocyte-derived. At 3 to 4 months of age the proportion of SF LMN cells expressing MHC class II was similar to that reported by Harkiss *et al* (1991) for adult sheep.

The reasons for the changes in MHC class II expression were not ascertained, but it has been established that macrophages from perinatal mice have an impaired antigen-presenting function because of suppression of MHC class II expression (Lu *et al* 1979). This is due to α-fetoprotein in serum that suppresses MHC class II expression for up to several weeks after birth (Lu *et al* 1984). However, local factors play an important role in determining MHC class II expression during this stage of development because the proportions of macrophages expressing these antigens was found to vary between different organs (Lu *et al* 1980). The factors that may be responsible for activation of macrophages in joints were outlined in Chapter 1, section 8.2.. The relative paucity of lymphocytes in these joints would appear to rule out lymphocyte-mediated activation. The deposition of IC or bacterial cell wall products in synovium are more feasible explanations although increased numbers of SLC expressing MHC class II did not appear until after the acute inflammatory changes, characterised by increased numbers of neutrophils in the SF and synovium, seen in the first week of life. It is unclear whether the inflammatory changes present up to 1 month of age were related to the changes in MHC class II expression by the SLC. The cause of these inflammatory changes was not established. During the first few days of life there may be increased levels of circulating bacterial antigens derived from the gastrointestinal tract as a result of

transiently increased permeability and the development of the gut flora (Linton and Hinton 1990). If these were deposited in joints it could result in a transient synovitis (Stimpson *et al* 1986). Polyarthritis caused by *E. rhusiopathae* infection is not uncommon during the first week of life and subclinical infections of this organism may result in a low grade synovitis (Lamont 1978). Another possibility is that with weight-bearing and increased limb movement after birth, components from articular cartilage may be released which could activate macrophages locally (Goto *et al* 1988). It is likely that the combined effect of systemic and local factors, that change during development, modulate macrophage activation within joints. Interestingly, no MHC class II-expressing SLC were present until after birth in the synovia from rats (Izumi *et al* 1990) demonstrating that the phenomenon is not confined to sheep.

The main function of MHC class II is presentation of peptide antigens to helper T lymphocytes (Germain 1994). The smaller numbers of cells expressing these products and its reduced level of expression early in the neonatal period compared to later in life suggests that the antigen-presenting capabilities of cells in joints at this time is reduced. This may result in joint tissues being more susceptible to infections early in neonatal life. The proportion of cells expressing MHC class II in synovium from the older lambs and adult sheep is not dissimilar to that described for normal synovium from humans (Palmer *et al* 1985) but is considerably greater than that described for the rat (Wilder *et al* 1987, Izumi *et al* 1990) and mouse (Klareskog *et al* 1982). The reasons for these differences are not clear.

In parallel with the observations made of MHC class II expression, the numbers of cells in the SLC layer that stained for NSE was very small in synovia from the foetuses compared to later in development. This result was a little suprising in view of the numbers of CD14-expressing cells in the synovium most of which would have been expected to stain positively (Yam *et al* 1971) and further suggested that these cells were in a state of functional immaturity.

The proportion of SLC expressing CD14 and FcγRII appeared to vary little with age with the majority of cells expressing these antigens at all time points. For CD14 this was reflected in the minor variation in the proportion of SF LMN cells expressing this antigen. The existence of FcR on SF LMN cells in sheep has previously been suggested by Harkiss *et al* (1991) who demonstrated enhanced antigen uptake in the presence of antigen-specific antibody *in vitro*, and all 3 classes of FcγR have been shown to be expressed by synovium from a variety of arthropathies in humans (Broker *et al* 1990). The expression of FcγRII by ovine synovium would appear to be very similar to that observed in human synovium. The cells in the SI expressing FcγRII were most likely to be macrophages although the receptor is also expressed by mast cells (Ravetch 1994), small numbers of which are present in ovine synovium (Chapter 4).

CD11c is expressed primarily by macrophages in sheep, although afferent lymph DC express low levels of this antigen (Gupta *et al* 1993). Because of its ability to bind the complement protein iC3b, CD11c has been termed complement receptor type 4 (Erdei *et al* 1991). It has been implicated in a number of cell adhesion-dependent processes including phagocytosis and chemotaxis (Larsen and Springer 1990). The numbers of cells expressing this antigen in ovine synovium would appear to be higher than that present in synovium from normal joints of humans where CD11c-expressing cells are either absent or occasionally observed (Allen *et al* 1989). The cells expressing this antigen in the SI were most likely to be macrophages.

Only small numbers of T lymphocytes were present in synovia from these sheep. The absence of DU2104+ B lymphocytes may have been due to reasons other than an absence of B lymphocytes from these tissues. Although Mab DU2104 has been reported to recognise a molecule expressed on all B cells of sheep (Mackay *et al* 1992b), and it does recognise a similar number of lymphocytes in PB compared to those expressing Ig (Dr. P. Bird, personal

communication), the Mab has not been fully characterised. If the expression of the antigen recognised by this Mab is down-regulated on B cells in tissues, this could explain their apparant paucity in synovium. However, DU2104+ B lymphocytes were detected in the SF and AL of adult sheep (Chapters 4 and 6) demonstrating that it is expressed on at least some B cells that have left the peripheral circulation. In most immunohistological studies of synovium, B lymphocytes have been identified using Mab to Ig (Duke *et al* 1982, Lindblad *et al* 1983). In normal human tissues the number of Ig-expressing cells has been reported to be low compared to the numbers of T lymphocytes (Lindblad and Hedfors 1987) which together with these findings and those in Chapter 4 suggest that there may be greater trafficking of T lymphocytes through normal joint tissues compared to B lymphocytes.

The results of this Chapter provided useful background information on joint tissues for the subsequent investigations described in the following Chapters.

CHAPTER 4

Pathological investigation of joints of sheep infected with Maedi-Visna virus

Introduction

Maedi-Visna virus (MVV) is a non-oncogenic retrovirus that causes degenerative and chronic inflammatory lesions in primarily the lungs (Maedi) and central nervous system (Visna) of sheep but also to a lesser extent in the mammary glands and joints (Oliver *et al* 1981, Watt *et al* 1992a). The commonest clinical signs of disease are dyspnoea, due to advanced pathological changes in the lung (chronic interstitial pneumonia characterised by lymphoid follicle development and smooth muscle hyperplasia), and weight loss (Watt *et al* 1992a). However, chronic inflammatory disease at the above sites frequently remains subclinical throughout the life of the animal and therefore may remain undetected (Haase 1986). Chronic inflammatory joint disease associated with natural MVV infection has been infrequently reported (Table 1) although there are several reports of experimental infection with the virus resulting in joint disease (Table 2). This is in contrast to CAEV infection of goats where up to 30% of naturally-infected animals are reported to show clinical signs of joint disease with the carpal, tarsal and stifle joints most commonly affected (Crawford and Adams 1981, Woodard *et al* 1982). Only a small proportion of these animals (under 5% of infected animals) are reported to develop severe erosive joint disease (Banks *et al* 1989). Although the incidence of clinical arthritis associated with naturally-occuring MVV infection is reported to be low (Table 1), joint disease in flocks of sheep can be easily overlooked, particularly if the disease is mild. In this respect there is some conflicting evidence in the literature with some authors suggesting that subclinical joint disease is absent whilst others suggest quite a high incidence (Table 1).

The immunopathogenesis of chronic inflammatory joint disease associated with MVV and CAEV infection is poorly understood. Several immunopathological studies have characterised the lymphocytic infiltrate in the joint tissues of these animals. Immunohistological studies of synovium from two arthritic MVV-infected sheep showed that the predominant T lymphocyte population was CD8+ (Kennedy-Stoskopf *et al* 1989). A similar finding was reported from the SF of two

Table 1. Summary of the literature describing arthritis in sheep naturally-infected with MVV.

Virus[a]	Breed	Clinical joint disease[b]	Joints affected[c]	Joint pathology[d]	Reference
OPPV	Border Leicester	6/18 (33%)	C or T	Advanced erosive changes in some joints, synovial hyperplasia, L, PC, necrosis and mineralisation, vasculitis (4 of 8 tissues).	Cutlip *et al* (1985a, b)
OPPV	Mixed breeds	2/13 (15%)	C	Erosive changes in both sheep. Synovial histology as above	Oliver *et. al.* (1981)
MVV	Texel	2/185 (1%). Lameness reported in >30% sheep*	C	31 RC examined at PM. 54% mild proliferative synovitis, 6% moderate to severe synovitis with GC-like structures.	Watt *et. al.* (1992a) Harkiss *et. al.* (1991)
MVV	Texel	0/24		Synovium from 1 RC of each sheep was examined. No evidence of synovitis.	Houwers *et. al.*(1987)
MVV	Oldenbred	0/45		No joint tissues examined.	Markson *et. al.* (1983)

[a] OPPV- Ovine Progressive Pneumonia virus is the term that is used to describe the North American strain of MVV

[b] The first figure shows the number of arthritic sheep and the second figure shows the number of sheep examined in the study

[c] C- carpal joints, T- tarsal joints

[d] L- lymphocytes, PC- plasma cells, GC- germinal centre, RC- radiocarpal joint

* cause of lameness not established

Table 2. Summary of the literature describing arthritis associated with experimental MVV infection

Virus[a]	Breed	Route[b]	Clinical joint disease[c]	Joints affected[d]	Joint pathology[e]	Reference
OPPV	Border Leicester	i/v or i/p	8/11 (83%)	C or T. C more frequently than T	Similar to descriptions for natural disease in the same study (Table 1)	Cutlip *et. al.* (1985a)
OPPV	Suffolk (<12mo)	i/a (RC)	4/4	C	Proliferative synovitis, L, PC, GC at day 42 post-infection	Oliver *et. al.* (1981)
		i/c or i/t	0/8		Moderate synovitis, L, PC in 3 of 8 synovia from the RC joints.	
OPPV (lytic and non-lytic strains)	Suffolk (<48hrs old)	i/t (lytic)	1/12 (8%)	C	Moderate synovitis, L, PC in 3 of 10 synovia from the RC joint, upto 43 wks post infection	Lairmore *et. al.* (1988a)
		i/t (non-lytic)	0/12		Synovium normal in all sheep	
MVV (3 strains)	Norwegian/ Icelandic	i/a (RC)	0/24		Moderate to severe synovitis, L, PC, and GC. No differences in severity between the 3 strains.	Larsen *et. al.* (1985)

[a] the terms lytic and non-lytic refer to the character of virus growth in tissue culture

[b] i/v- intravenous, i/p- intraperitoneal, i/t- intratracheal, i/a- intraarticular, RC- radiocarpal joint

[c] The first figure refers to the number of sheep with visible joint enlargement and the second figure shows the number of sheep in the experiment.

[d] C- carpal joint, T- tarsal joint

[e] L- lymphocytes, PC- plasma cells, GC- germinal centres

arthritic CAEV-infected goats (Wilkerson *et al* 1993). However, Harkiss *et al* (1991) showed that CD8+ T lymphocytes were predominant in SF from some joints of a group of MVV-infected sheep, most of which were not showing clinical signs of arthritis. Additionally, an increased proportion of SF macrophages from the joints of these sheep expressed MHC class II and at a higher intensity compared to SF macrophages from control sheep. If chronic inflammatory changes are present in the joint tissues of sheep in the absence of overt clinical joint disease, their characterisation may provide a valuable insight into the pathogenesis of lentiviral arthritis.

The aim of this study was to characterise the gross, histological and immunopathological features of joint tissues from MVV-infected sheep with and without clinical signs of joint disease. These results were compared with joint tissues from non-infected control sheep.

Outline of Methods

The MVV-infected sheep described in this study were from a flock naturally-infected with the virus, that fluctuated in number from 50 to approximately 90. In addition, sheep experimentally-infected with the virus that were maintained by other groups within the Department of Veterinary Pathology, Edinburgh, were investigated if they developed swollen joints. Sheep were defined as clinically arthritic or non-clinically arthritic according to the criteria outlined in Chapter 2, section 3.1.. Control sheep were obtained from a flock with no history of MVV infection, were seronegative for the virus by AGID and were non-clinically arthritic.

Most of the results described in this Chapter were obtained following examination of sheep at post mortem. The majority of these sheep were euthanased electively either because of deterioration in their clinical condition or to reduce sheep numbers in the flock. Following euthanasia, all limb joints in all sheep were examined grossly. For clinically arthritic MVV-infected sheep, an internal joint

examination was performed on all grossly enlarged joints following aspiration of SF. Where arthritis affected joints unilaterally, the same joint of the contralateral limb was examined internally. For non-clinically arthritic MVV-infected and control sheep, SF was aspirated from the RC and TT joints, prior to the internal examination of these joints. These joints were chosen for examination because of their predisposition to develop arthritis. For all joints examined internally, the gross appearance of the synovium and articular cartilage was recorded and graded according to the descriptions detailed in Chapter 2, section 4.. Portions of synovium from the RC and TT joints were fixed in 10% buffered formol-saline and the remainder was snap frozen and stored at -70^{0}C. Additionally, synovium from several sheep used for the immunohistological studies was obtained by open biopsy under general anaesthesia (see Chapter 2, section 14.2.).

To determine whether the proportions of non-clinically arthritic MVV-infected and control sheep differed with respect to gross pathological changes in their joints, chi-squared (χ^2) tests were performed. Synovial histology was evaluated from sections stained using standard techniques (H+E, MPG, and toluidine blue). Immunocytochemical characterisation of synovia was performed using a range of Mab to B and T lymphocyte subsets (CD4+, CD8+ and $\gamma\delta$), MHC class II (DRβ and DQβ antigens), CD1 and the viral core protein P15. The densities of the T lymphocyte subsets and proportions of the MHC class II-expressing cells (SLC and cells in the SI were evaluated separately) was determined as described in Chapter 2, section 6.4.3.. CD4:CD8 and $\alpha\beta$:$\gamma\delta$ T lymphocyte ratios were calculated from the T cell densities. In a small number of clinically arthritic MVV-infected sheep, the presence of MVV in synovium was investigated by culturing synovial explants *in vitro* (Chapter 2, section 6.5.).

TNCC and differential cell counts of SF were obtained following aspiration of SF at post mortem, and in separate experiments on non-clinically arthritic MVV-infected and control sheep (SF aspiration was performed with sheep sedated as described in Chapter 2, section 2.3.1.), the proportions of the B and T lymphocyte

subsets in SF was determined by flow cytometry. Non-parametric statistical analysis was performed using Mann Whitney U-tests, and Wilcoxon Signed Rank tests where data was paired.

Results

1. The incidence of clinical arthritis in a flock of MVV-infected sheep

Over a 4 year period, 10 sheep (approximately 11% of the original flock) from the flock developed clinical arthritis that affected at least one limb joint. Additionally, 2 of approximately 40 sheep experimentally-infected (approximately 10^5 $TCID_{50}$ of MVV strain EV1 administered subcutaneously, a minimum of 2.5 years prior to the detection of joint disease) with the virus developed clinical arthritis (both developed moderate bilateral carpal joint enlargement). The breeds of these sheep are shown in Table 3. All arthritic sheep were female and aged at least 4 years. In 10 of these 12 arthritic sheep, joint disease was confined uni- or bilaterally to the carpal joints (Table 4 and 5). One sheep developed polyarthritis that affected both elbows and stifles and one carpal joint, and the remaining sheep had bilaterally affected elbow joints. Lameness was detectable in only 3 (25%) of these sheep.

2. Post mortem findings

The numbers of sheep examined at post mortem and the breed distribution is shown in Table 3. All sheep were female and aged at least 4 years.

2.1. Post mortem findings in non-clinically arthritic MVV-infected and control sheep.

Mild synovitis was present in a proportion of the RC and TT joints of both groups of sheep. 7 MVV-infected (24%) and 4 control sheep (17%) had a mild synovitis of 1 RC joint, and 1 MVV-infected sheep had a mild synovitis of 1 TT joint. Minor defects of the articular cartilage were present in one or both RC joints

Table 3. Breed distribution of MVV-infected and control sheep

MVV-infected				Control	
A. Clinically arthritic		B. Non-clinically arthritic			
Breed	No.	Breed	No.	Breed	No.
Texel or Texel X	10 (83%)	Texel	26 (90%)	Blackface or	15 (65%)
Finn or Finn X	2 (17%)	Mule	1 (3%)	Blackface X	
		Cotswold	1 (3%)	Cheviot X	6 (26%)
		Kerry Hill	1 (3%)	Dorset Horn X	1 (4%)
				Texel	1 (4%)
Total	12		29		23

Table 4. Frequency of affected joints in clinically arthritic MVV-infected sheep

	Carpus		Elbow	Stifle
	Unilateral	Bilateral	Bilateral	Bilateral
Number of clinically arthritic sheep (n=12)	6	5	2	1

Table 5. Number of affected joints in clinically arthritic sheep

	Number of affected joints		
	1	2	>2
Number of clinically arthritic sheep (n=12)	5	6	1

in 45% of the MVV-infected and 52% of the control sheep. These values for the TT joint were 38% and 48% respectively. Statistical comparison of proportions (χ^2 test) showed that there was no significant differences between MVV-infected and control sheep. However, the proportion of sheep with mild synovitis in their RC joints was significantly higher than the proportion of TT joints with these changes ($p<0.025$ and <0.05 for MVV-infected and control sheep respectively). No sheep from either group had erosions of the articular cartilage in any joint. However, mild superficial abrasions and partial thickness defects of the articular cartilage were common in RC and TT joints from both groups of sheep.

2.2. Post mortem findings of clinically arthritic joints from MVV-infected sheep

These are summarised in Table 6. Synovitis was present in all clinically arthritic joints with the exception of those in sheep 59 and 91. In the more severe cases the synovium was tan-coloured and showed signs of petechiation (Figure 1a). Severe cartilage erosion (grade +++) with pannus extending across the articular surface was present unilaterally in the RC joints of 2 sheep (9% of arthritic joints) (Figure 1b). Two sheep with bilaterally arthritic elbow and stifle joints (Sheep 57 and 80 respectively) had erosions on the weight-bearing aspects of the articular cartilage of the distal humerus and distal femur respectively but these were not associated with the development of pannus. Mild or moderate erosions at the chondrosynovial junction along the anterior margin of the carpal bones (Figure 1a) were present in 8 of 14 (57%) of the remaining clinically arthritic RC joints. Overall, 56% of clinically arthritic joints showed some degree of articular cartilage erosion.

3. Synovial histology

Synovial histology was evaluated from sections of formol-fixed tissues. The numbers of layers of cells comprising the SLC layer and the degree of lymphocytic

Table 6. Summary of gross post mortem findings and synovial histology in clinically arthritic MVV-infected sheep.

Number of arthritic joints	Sheep No.[a]	Joint[b]	Joint enlargement[c]	Cartilage erosion[d]	Severity of synovial inflammation[e]
1	59	LC	+	-	+/(-)
	79	LC	++	-	+/(+)
	1078*	RC	++	+	++/(+)
	003	RC	+++	+++	+++/(+)
2	91	RC/LC	+/+	-/-	-/-
	89	RC/LC	+/+	-/-	+/+
	30	RC/LC	+/+	+/+	++/++
	848	RC/LC	++/++	++/+	++/++
	1550*	RC/LC	++/++	+/+	+++/+++
	013	RC/LC	+++/+	++/-	++/+
	57	RE/LE	+++/+++	+/+	-/-
>2	80	LC	++	-	++
		RE/LE	++/++	-/-	NK
		RS/LS	+++/+++	++/++	+++/+++

[a] * denotes experimentally-infected sheep. All sheep were of the Texel breed except for 1078 and 1550 which were of the Finn breed.

[b] R-right, L-left, C-radiocarpal, E-elbow, S-stifle

[c] + mild, ++ moderate, +++ severe

[d] - absent, + mild, ++ moderate, +++ severe

[e] Graded according to the degree of lymphoid infiltration detailed in Chapter 2, section 6.4.1.. The score in parentheses is that obtained for synovium from the contralateral joint.

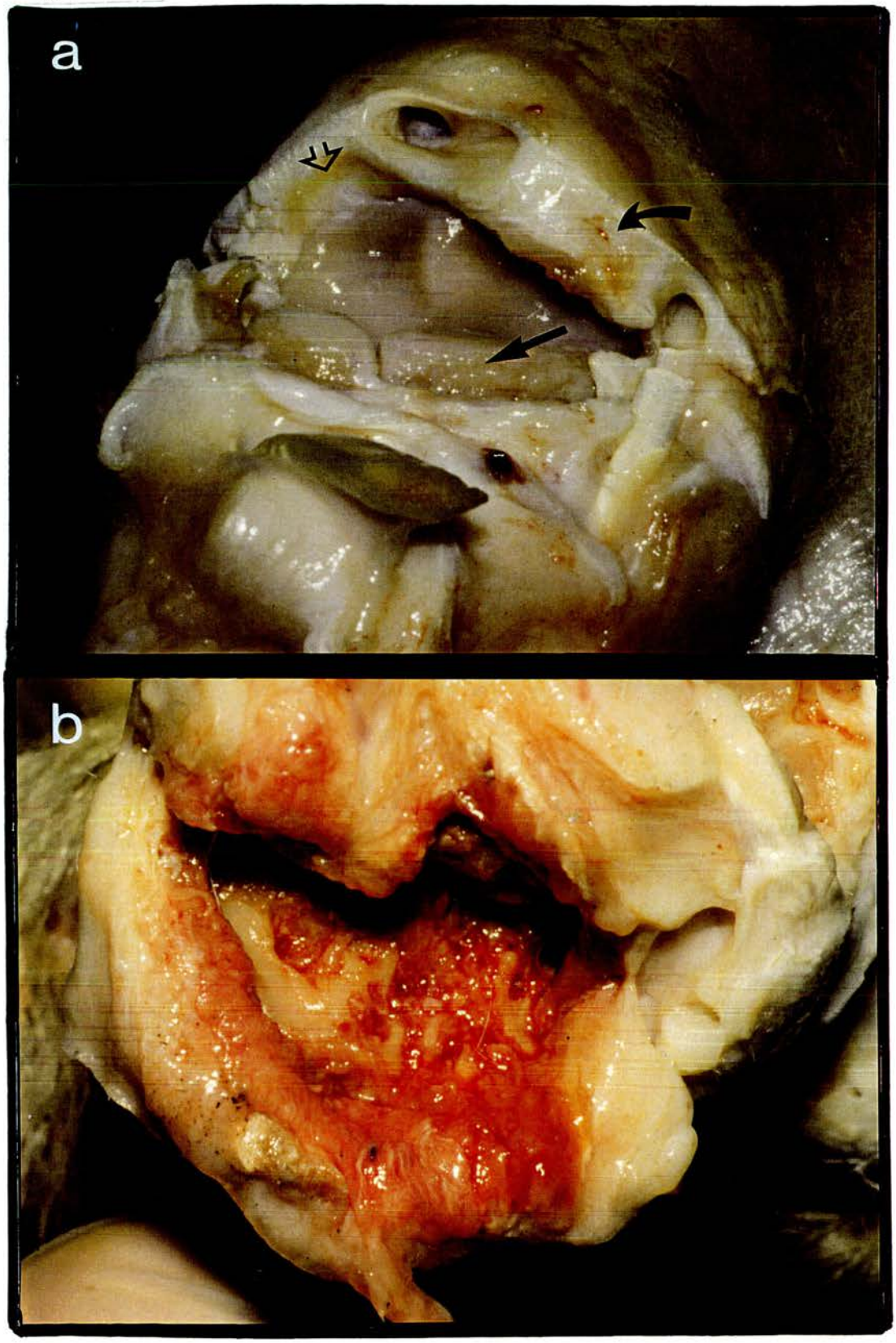
NK-not known

Figure 1. Clinically arthritic joints from MVV-infected sheep at post mortem.

(a). RC joint from sheep 1550 (Table 7). The joint has a moderately thickened joint capsule (++)(curved arrow), tan-coloured and thickened synovial lining (open arrow) and mild erosions (+) at the chondrosynovial junction along the anterior edge of the radial and ulnar carpal bones (black arrow).

(b). RC from sheep 003 (Table 7). The articular cartilaginous surfaces of the joint have been destroyed by the invasion of pannus.

Figure 1



infiltration were graded using the classification system detailed in Chapter 2, section 3.1. These results for control and MVV-infected sheep are shown in Table 7.

3.1. Synovium from non-clinically arthritic MVV-infected and control sheep

Synovium from one RC and one TT joint from 15 and 29 of the above mentioned control and non-clinically arthritic MVV-infected sheep respectively, was examined histologically. Synovium from the RC joints was generally areolar in character (Figure 2) whilst that from the TT joint was adipose. χ^2 analysis of proportions showed that there was no significant difference in the proportion of synovia from the RC or TT joints with SLC hyperplasia or lymphoid infiltrates (mild and moderate grades combined) when MVV-infected and control sheep were compared. Small numbers of plasma cells were present in a minority of synovia from the RC and TT joints of both groups of sheep. Also, small numbers of mast cells were present diffusely scattered throughout the synovium from both joints of both groups of sheep. Although not quantitated, their numbers in synovia from both groups of sheep appeared to be similar. Large numbers of LMN were present as the predominant cell type in synovia from 1 RC and 1 TT joint from the MVV-infected sheep (different animals) and in both cases these cells greatly exceeded the numbers of lymphocytes present (Figure 2). In synovia from 2 RC and 1 TT joints from MVV-infected sheep, smaller arteriolar vessels were observed with marked fibromuscular hyperplasia of their intimal linings (Figure 2). Only one or two such vessels were observed in each section but blood vessels with this appearance were not seen in synovia from control sheep.

3.2. Synovia from clinically arthritic MVV-infected sheep

Synovia from 21 clinically arthritic joints from 12 sheep were examined. Synovia from the affected joints of all arthritic animals, with the exception of sheep 57 and 91, showed varying degrees of chronic inflammation characterised by a mild

Table 7. Summary of histological changes in synovia from MVV-infected and control sheep.

		Joint[a]	SLC hyperplasia[b] (%)				Lymphoid infiltrate[c] (%)			
			-	+	++	+++	-	+	++	+++
Control sheep		RC (15/15)	53	47	-	-	80	13	7	-
		TT (15/15)	70	30	-	-	85	8	7	-
MVV-infected sheep	A. Non-clinically arthritic	RC (29/29)	31	69	-	-	62	28	10	-
		TT (29/29)	23	77	-	-	66	28	6	-
	B. Clinically arthritic.									
	i. Inflammatory	(18/10)	27	55	18	-	-	27	54	19
	ii. Non-inflammatory	(4/2)	-	100	-	-	100	-	-	-

[a] The numbers in parentheses indicate the number of synovia examined (first figure) from the number of sheep (second figure)

[b] The thickness of the SLC layer was graded according to the scale outlined in Chapter 2, section 6.4.1.. Briefly, the thickness of this layer was graded as normal (-) or increased to a mild, moderate or severe degree (+ to +++).

[c] The severity of the lymphoid infiltrate was graded according to the method outlined in Chapter 2, section 6.4.1.. Briefly, lymphoid cells were absent (-) or present in increased numbers (+ to +++, depending on severity of infiltrate).

Figure 2. Histological appearance of synovium from control and MVV-infected sheep.

(a). Synovium from a RC joint of a control sheep (H+E, original magnification x156). The synovium is areolar in character.

(b). Synovium from the RC joint of a clinically arthritic MVV-infected sheep (number 1550, see Table 7)(H+E, original magnification x156). There is no thickening of the SLC layer on this part of the section but the SI is densely vascularised and LMN are present in abundance. Small numbers of lymphoid cells are visible in a diffuse band beneath the SLC layer (overall, the section was graded ++ for lymphoid infiltrate).

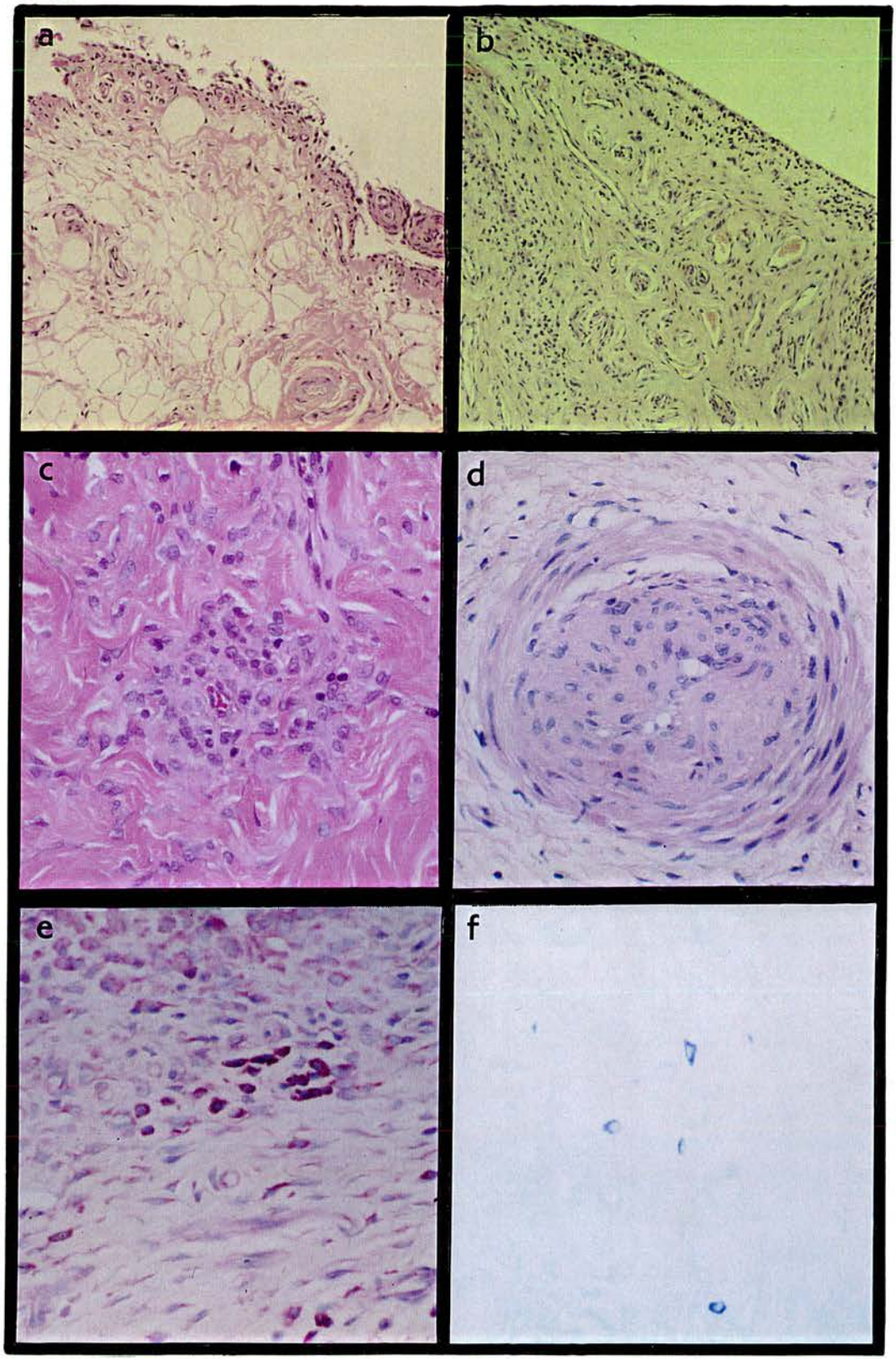
(c). Synovium from a RC joint of a non-clinically arthritic MVV-infected sheep (H+E, original magnification x390). Large numbers of LMN are present in the SI.

(d). Synovium from a RC joint of a non-clinically arthritic MVV-infected sheep (H+E, original magnification x25). The blood vessel has marked fibromuscular hyperplasia of its intimal lining. Vessels with this appearance were only seen in synovia from MVV-infected sheep.

(e). Synovium from a RC joint of a clinically arthritic MVV-infected sheep (MPG, original magnification x390). Small numbers of plasma cells are present.

(f). Synovium from a RC joint of a clinically arthritic MVV-infected sheep (toluidine blue, original magnification, x390). Small numbers of mast cells are scattered throughout the SI.

Figure 2



or moderate increase in the thickness of the SLC layer, increased vascularity, fibrosis and infiltration by lymphocytes and LMN (Table 7). Synovia from the affected joints of 2 sheep were richly vascularised with very large numbers of small blood vessels (Figure 2). LMN cells were predominant in these synovia and their numbers greatly exceeded the numbers of lymphoid cells. Blood vessels of the appearance described in section 3.2. were observed in synovia from 7 joints (33% of clinically arthritic joints). Small to moderate numbers of plasma cells were present in all chronically inflamed synovia together with small numbers of mast cells (Figure 2). There were no obvious differences in the histological appearance of tissues from naturally-infected compared to experimentally-infected sheep. Minimal inflammatory changes were present in synovia from sheep 57 and 91 and the TNCC of SF from these joints (all under 3.3 $x10^5$/ml) were within the range of values obtained for TNCC of SF from the RC joints of control sheep. The relative absence of inflammatory changes affecting synovial tissues from these sheep suggested that these sheep had non-inflammatory joint disease.

4. Characterisation of synovium by immunocytochemistry

Synovium from one RC and one TT joint were randomly selected from 10 control and 8 non-clinically arthritic MVV-infected sheep and evaluated by immunocytochemistry. Additionally, synovium from 7 clinically arthritic joints (6 RC and 1 stifle) from 7 MVV-infected sheep with chronic inflammatory arthritis were evaluated. Because of the relative paucity of lymphocytes in synovia from non-arthritic sheep (MVV-infected and control) it was not possible to calculate CD4:CD8 and αβ:γδ T cell ratios for all joints. Statistical comparisons between groups of sheep were performed where sufficient numbers of values were obtained.

4.1. Synovium from the RC and TT joints of control sheep

The majority of SLC in synovia from these joints expressed MHC class II DR and DQ antigens (Figure 3 and 4). The deeper cells of the SLC layer did not

Figure 3. Immunohistology of synovium (Immunoperoxidase technique).

(a)(b)(c) and (d). Synovium from the RC joints of clinically arthritic (a and b), non-clinically arthritic MVV-infected (c) and control sheep (d) labelled with Mab to MHC class II (DR)(original magnification x390). Virtually all of the lymphocytes in the perivascular cuff in section (a) are positively-stained and very large numbers of positively-stained cells (mainly LMN) surround the two blood vessels with fibromuscular hyperplasia of their intimal linings in section (b). Most of the cells in the SLC layer in section (c) are positively-stained and large numbers of cells in the SI, including endothelial cells, are labelled with the Mab. The most superficial cells in the SLC layer in synovium from the control sheep (d) are positively-stained but the cells in the deeper areas of this layer have not been labelled with the Mab. No positively-stained cells in the SI are visible on this area of the section.

(e) and (f). Synovium from the RC and stifle joints of a clinically arthritic MVV-infected sheep labelled with Mab to CD1 and the viral core protein P15 respectively (original magnification x390). Quite large numbers of positively-stained cells are present in the SI in section (e), some of which have long cytoplasmic processes. A single P15-expressing cell is present in section (f).

Figure 3

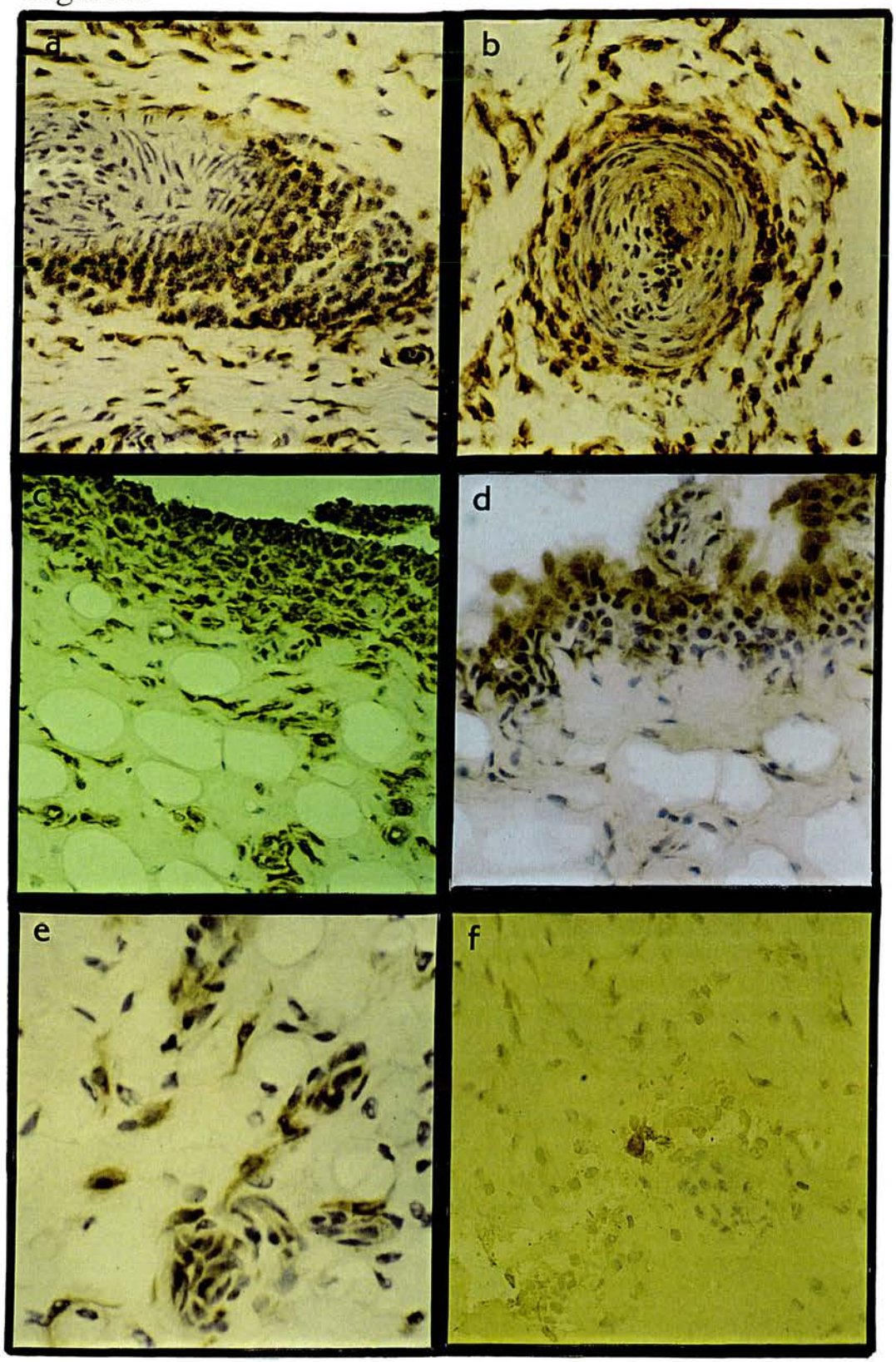


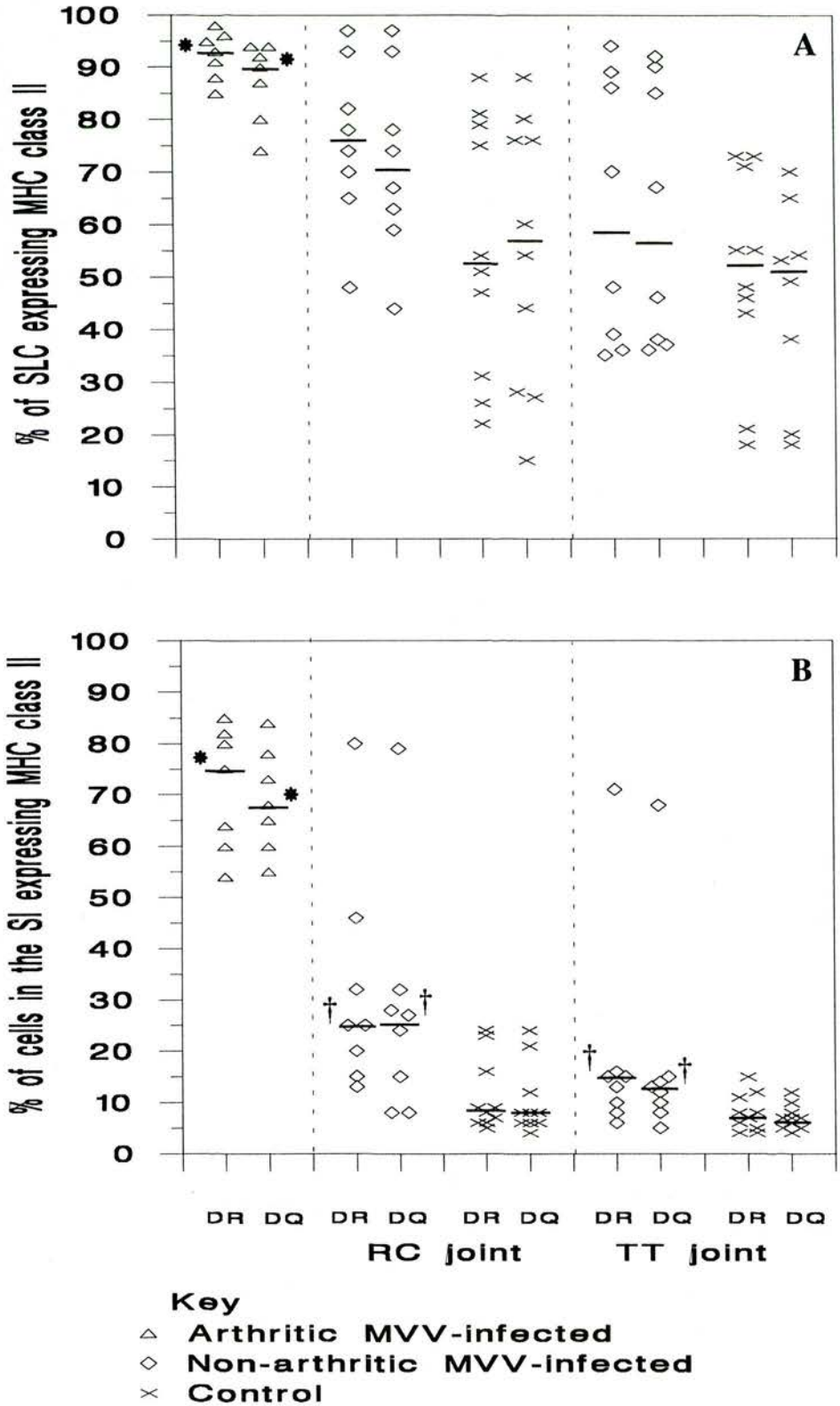
Figure 4. Quantitation of MHC class II expression by SLC (A) and cells in the SI (B) of synovia from the RC and TT joints of MVV-infected and control sheep.

The proportion of MHC class II-expressing cells (DR and DQ) in synovia from clinically arthritic (n=7), non-clinically arthritic MVV-infected (n=8) and control sheep (n=10) was determined by counting 500 cells in the SLC and SI layers in identical areas of sequential sections labelled with Mab to the above antigens (Immunoperoxidase technique). The median values are shown by horizontal bars.

For graph A, *$p<0.001$ compared to values from control sheep.

For graph B, *$p<0.01$ compared to values from non-clinically arthritic MVV-infected sheep. $^{\dagger}p<0.05$ compared to values from control sheep.

Figure 4


A
% of SLC expressing MHC class II
100
90
80
70
60
50
40
30
20
10
0
B
% of cells in the SI expressing MHC class II
100
90
80
70
60
50
40
30
20
10
0
DR DQ DR DQ DR DQ DR DQ DR DQ
RC joint
TT joint
Key
Arthritic MVV-infected
Non-arthritic MVV-infected
Control

usually express either of these antigens, regardless of its overall thickness. Small numbers of MHC class II-expressing cells were present in the SI and morphologically these resembled lymphocytes and LMN cells. The numbers of blood vessels with endothelial linings expressing MHC class II was highly variable. Only occasional weakly-stained CD1-expressing cells were present in the SLC layer and in the SI.

Small numbers of T lymphocytes were present in synovia from 9 of 10 RC joints and all synovia from the TT joints. CD4+ T cells predominated over the other T lymphocyte subsets in synovia from the RC joint whereas the reverse was true for synovia from the TT joint (The median CD4:CD8 ratios in synovia from the RC and TT joints were 1.5 and 0.4 respectively). DU2104+ B lymphocytes were rarely observed in tissues from either joint. For synovia from the RC joints, the densities of CD4+ and CD8+ T cells were significantly higher ($p<0.03$ for both subsets) than the γδ T cells but this was not the case for synovia from the TT joints (Figure 5.1). Also, the densities of each subset were significantly higher in synovia from the RC joints than paired TT joints ($p<0.04$ for all 3 subsets). The majority of T lymphocytes of all three subsets were diffusely scattered throughout the synovia, except for tissue from one RC joint where several small focal and perivascular aggregates of CD4+ T cells were present. No P15-expressing cells were observed in any tissues.

4.2. Synovia from the RC and TT joints of non-clinically arthritic MVV-infected sheep.

Although an increased proportion of the SLC expressed MHC class II antigens compared to control tissues, the differences were not statistically significant (Figure 4). However, there was a significant increase in the number of MHC class II-expressing cells in the SI of synovia from the RC joints compared to paired TT joints ($p<0.05$ for both reagents), and compared to synovia from control sheep ($p<0.01$ for both reagents in the RC and TT joints)(Figure 3 and 4).

Figure 5.1 and 5.2. Densities of T lymphocytes in synovia from MVV-infected and control sheep.

Figure 5.1. The densities of CD4+, CD8+ and γδ T lymphocytes in synovia from the RC (a) and TT joints (b) of non-clinically arthritic MVV-infected and control sheep (n=8 and 10 respectively) were obtained by counting the number of positively-stained cells in identical areas of sequential sections (Immunoperoxidase technique). A minimum area of $2mm^2$ was assessed on each section. The median values are indicated by horizontal bars. (*$p<0.04$ compared to values from control sheep). To show all values obtained using this method on the graph, 1 was added to each result.

Figure 5.2. Densities of T lymphocyte subsets in synovia from clinically arthritic MVV-infected sheep (n=7). These were calculated in the same way as described above.The median values are indicated by horizontal bars. The densities of all 3 subsets in synovia from clinically arthritic MVV-infected sheep was significantly higher than non-clinically arthritic MVV-infected or control sheep ($p<0.004$ and <0.001 for all 3 subsets compared to these groups respectively).

Figure 5.1

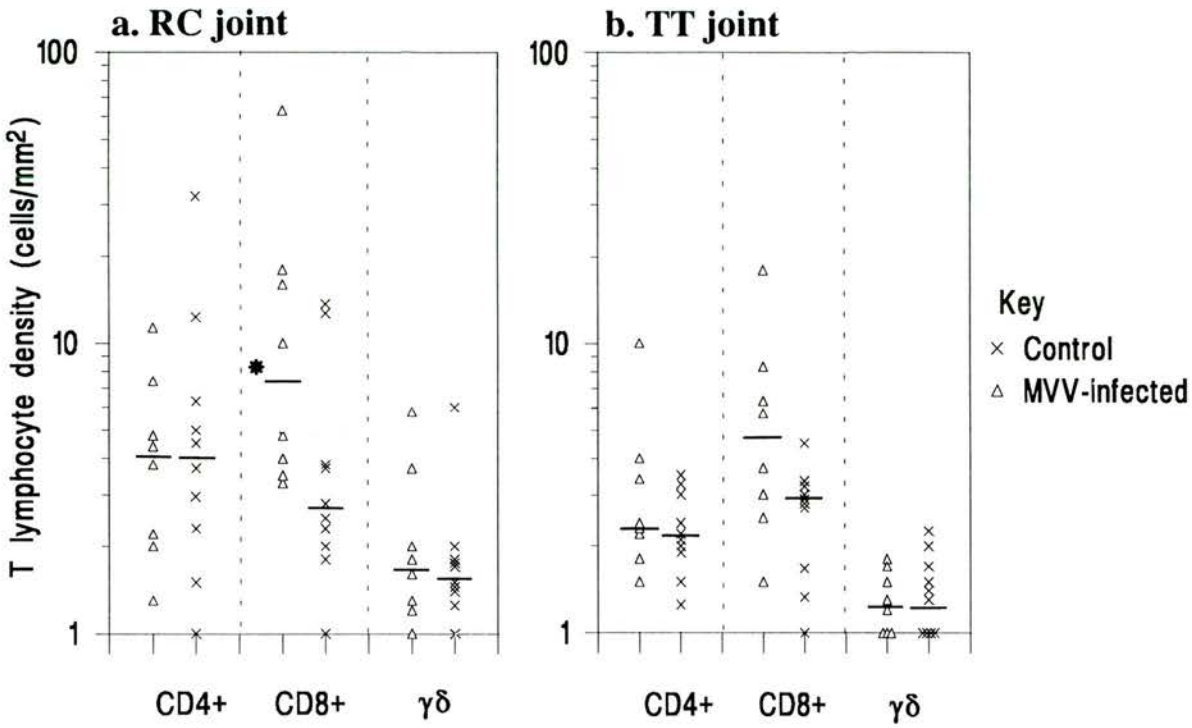
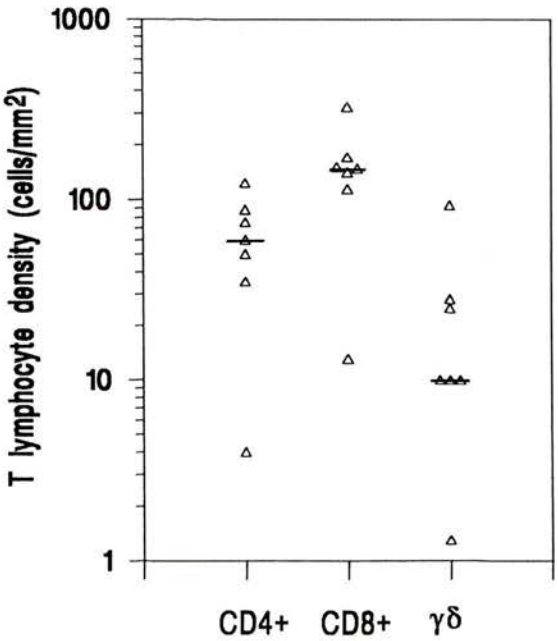


Figure 5.2



Morphologically, most positively-stained cells resembled LMN cells and to a lesser extent lymphocytes. Additionally, many of the endothelial linings of blood vessels expressed MHC class II. Small numbers of CD1-expressing cells in the SLC layer and SI were present in some sections.

Although the densities of all three T lymphocyte subsets were generally higher than those present in tissues from control sheep, the differences were only statistically significant for the CD8+ T lymphocyte subset in synovia from the RC joint ($p<0.04$)(Figure 5.1). This resulted in a significant lowering of the CD4:CD8 ratio ($p<0.009$) compared to that from the RC joints of control sheep (median CD4:CD8 ratios from these joints were 1.5 and 0.3 for control and MVV-infected sheep respectively). Although most of these cells were diffusely scattered immediately beneath the SLC, small focal and perivascular aggregates were present in some tissues. The distribution of CD4+ and γδ T cells was similar to that observed in tissues from control sheep. Very few DU2104+ B lymphocytes and no P15-expressing cells were present in synovia from either joint.

4.3. Synovia from clinically arthritic MVV-infected sheep

A high proportion of cells in the SLC and SI layers expressed MHC class II DR and DQ antigens (Figure 4). For the SLC layer, a significantly higher proportion of cells expressed DR and DQ antigens compared to control tissues ($p<0.001$ for DR and DQ antigens) but not compared to tissues from non-clinically arthritic MVV-infected sheep. Also, a significantly higher proportion of the cells in the SI expressed both DR and DQ antigens compared to synovia from non-clinically arthritic MVV-infected sheep ($p<0.04$ for both antigens)(Figure 3 and 4). Positively-stained cells morphologically resembled LMN cells and lymphocytes and many of the endothelial cell linings of blood vessels expressed MHC class II, including those vessels with marked intimal fibromuscular hyperplasia (Figure 3). These latter vessels were often surrounded by large numbers of MHC class II-expressing cells, most of which resembled LMN cells. CD1 expression was variable

although quite large numbers of positively-stained cells were present in some tissues (Figure 3).

The densities of all three T lymphocyte subsets were significantly raised compared to synovia from the RC joints of non-arthritic MVV-infected and control sheep ($p<0.004$ and <0.001 for all three subsets from both groups of sheep respectively)(Figure 5.2). CD8+ T cells were the predominant T cell subset in all tissues. Large numbers of these cells were diffusely scattered beneath the SLC, and focal and perivascular aggregates were present in most tissues (Figure 6). Although the CD4:CD8 ratio (median ratio of 0.3) was not significantly different from non-clinically arthritic MVV-infected sheep, it was significantly lower than the ratios from RC joints of control sheep ($p<0.015$). CD4+ and γδ T cells were also scattered throughout the SI but there was a greater tendency for CD4+ T cells to be localised in focal or perivascular aggregates. There was no significant difference in the αβ:γδ T cell ratio comparing these tissues (median of 16) with those from the RC joints of non-clinically arthritic MVV-infected or control sheep (median in both groups was 10). Only very small numbers of DU2104+ B lymphocytes were diffusely scattered throughout the SI.

Occasional P15-expressing cells were present in synovia from 3 sheep (Figure 3). Morphologically, these cells resembled LMN cells.

5. SF analysis

SF TNCC were obtained from one or both RC and TT joints from each of 21 non-clinically arthritic MVV-infected and 15 control sheep. Also, SF was aspirated from 8 clinically arthritic joints from 6 sheep. No SF could be aspirated from a further 6 clinically arthritic joints from these sheep and subsequent post mortem examination confirmed that negligible quantities of SF were present in these joints. Differential cell counts were obtained from SF from these clinically arthritic sheep and from one or both RC and TT joints from 12 non-clinically arthritic MVV-infected and 12 control sheep. These results are shown in Table 8.

Figure 6. T Lymphocyte subsets in synovia from a clinically arthritic MVV-infected sheep (Immunoperoxidase technique, original magnification x156).

(a)(b) and (c). Sequential sections of synovium labelled with Mab to CD4 (a), CD8 (b) and γδ T cell receptor (c)(arrow indicates a positively-labelled cell). Most of the lymphocytes are diffusely scattered throughout the SI. (d) shows a perivascular cuff of CD8+ T lymphocytes deeper within the SI.

Figure 6

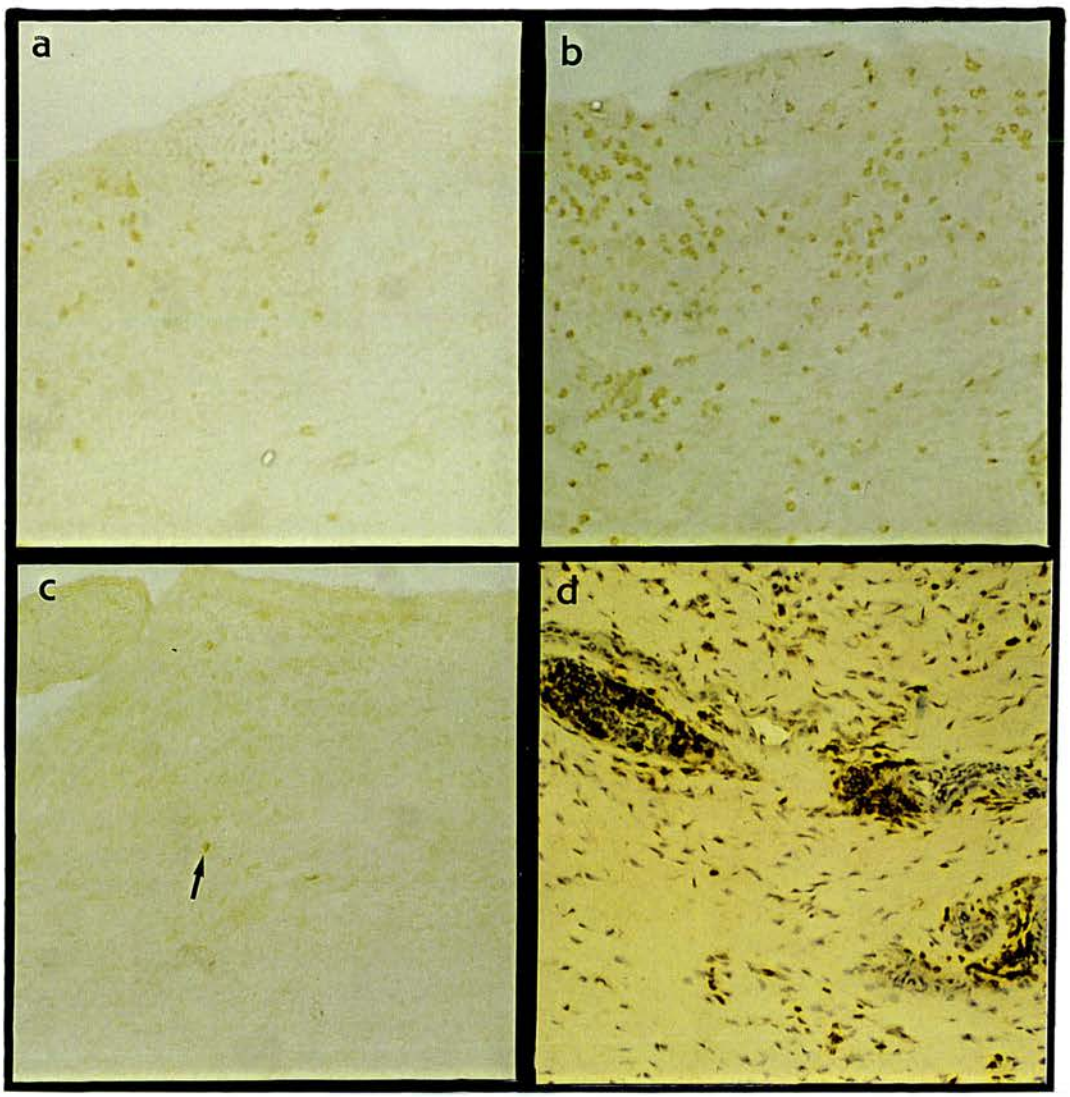


Table 8. Differential cell counts of synovial fluid from MVV-infected and control sheep

		Joint[a]	TNCC ($x10^5$/ml)	Neutrophils %	Lymphocytes %	Synoviocytes %	Macrophages %
MVV-infected	a. Clinically arthritic	RC (6/4)	49 ± 23† (25-73)	10 ± 10 (0-27)	40 ± 20 (15-74)	48 ± 17 (23-73)	2 ± 2 (0-5)
	b. Non-clinically arthritic	RC (19/19)	3.1 ± 4.8* (0.7-32)	5 ± 7 (0-23)	11 ± 12 (1-50)	82 ± 15 (36-99)	2 ± 2 (0-8)
		TT (19/19)	3.3 ± 4.8 (0.2-25)	26 ± 32 (0-92)	13 ± 8 (2-30)	54 ± 31 (5-99)	4 ± 8 (0-34)
Control		RC (15/15)	1.6 ± 1.1 (0.3-4.1)	20 ± 29 (0-87)	7 ± 4 (1-19)	68 ± 32 (9-97)	1 ± 1 (0-4)
		TT (15/15)	1.8 ± 1.5 (0.1-5.4)	28 ± 37 (0-91)	6 ± 9 (1-39)	69 ± 36 (5-98)	1 ± 1 (0-4)

Values shown are the mean ± s.d. with the range shown in parentheses below.

[a] The numbers in parentheses show the number of SF examined (first figure) from the number of sheep (second figure).

† $p<0.002$ compared to SF TNCC from RC joints of non-arthritic MVV-infected sheep

* $p<0.005$ compared to SF TNCC from RC joints of control sheep

5.1. TNCC and differential cell counts

There was no significant difference in the TNCC of SF from paired RC and TT joints from non-clinically arthritic MVV-infected and control sheep. SF TNCC from the RC joints, but not the TT joints, of non-clinically arthritic MVV-infected sheep were significantly raised compared to these joints from control sheep ($p<0.005$). Synoviocytes were the predominant cell type in SF from all joints. In addition to the 4 major cell types, occasional eosinophils, dividing cells and binucleate cells were observed in a minority of SF from both groups of sheep. They were always present at a very low frequency relative to the other cell types and their numbers were not quantitated.

The TNCC of SF from clinically arthritic sheep was significantly raised compared to SF from the RC joints of non-clinically arthritic MVV-infected sheep ($p<0.0013$). Lymphocytes and synoviocytes were the predominant cell types in these SF. Other cell types described above were occasionally observed.

6. Flow cytometric analysis of lymphocytes in PB and SF

Immunocytochemical evaluation of synovia from the RC joints (section 4.2.) suggested that higher numbers of CD8+ T lymphocytes were present in tissues of non-arthritic MVV-infected sheep compared to control sheep. To determine whether this was reflected in the proportions of the lymphocyte subsets in SF, these were evaluated by flow cytometry. The percentages of the lymphocyte subsets in SF from the RC and TT joints of 9 non-clinically arthritic MVV-infected and 9 control sheep (Blackface ewes aged a minimum of 4 years) was determined in the manner described in Chapter 2, section 11. The ratios of CD4:CD8, $\alpha\beta$:$\gamma\delta$ and T:B lymphocytes were calculated from these results and compared with these ratios in PB. These results were then compared with those obtained from a group of control sheep. PB and SF from the affected joints of 2 clinically arthritic MVV-infected sheep were also evaluated. For paired comparisons of PB and RC or TT joints, and for comparing paired RC and TT joints, the mean ratios for these joints

in each animal were used. Percentage values of lymphocyte subsets that were below 1% were regarded as insignificant. Because of the paucity of lymphocytes in SF of some sheep, it was not possible to calculate all the above lymphocyte ratios for all joints. The non-clinically arthritic MVV-infected and control sheep used in this experiment were different from those described in the above sections. The percentage values of the lymphocyte subsets are shown in Table 9 and typical FACS profiles of SF lymphocytes from the joints of both groups of sheep are shown in Figure 7.

6.1. Control sheep

The proportion of CD4+ and CD8+ T lymphocytes approximated to each other in the SF from RC and TT joints (Table 9). The CD4:CD8 ratios in SF from RC and TT joints were significantly lower than in paired PB ($p<0.02$ and <0.009 for the these joints respectively). Because of the paucity of γδ T and B lymphocytes in SF, for statistical analysis, the results from the RC and TT joints were combined for comparison with PB. The αβ:γδ T lymphocyte ratio was significantly lower in SF ($p<0.03$), and the T:B ratio was significantly higher in SF compared to paired PB ($p<0.0001$).

6.2.2. Non-clinically arthritic MVV-infected sheep

There was no significant difference in the CD4:CD8 or T:B ratio in PB from these sheep compared to the PB of control sheep. However, the αβ:γδ T lymphocyte ratio was significantly higher in the PB of the MVV-infected sheep compared to PB from control sheep ($p<0.006$).

CD8+ T lymphocytes predominated in the SF from RC and TT joints of these sheep. Similar to the control sheep, the CD4:CD8 ratio in SF from both joints was significantly lower than paired PB ($p<0.014$ for RC and TT joints). Also, the CD4:CD8 ratios in SF from RC and TT joints were significantly lower ($p<0.0001$ and <0.0006 for the RC and TT joints respectively) than the values obtained for

Figure 7. Flow cytometry profiles of lymphocytes in SF from the RC and TT joints of non-clinically arthritic MVV-infected (B) and control sheep (A).

Briefly, cells in SF from the RC and TT joints from these sheep were labelled with Mab to B and T lymphocyte subsets. For analysis the live gate shown in Chapter 2, Figure 3.1. was used. 1000 to 2000 cells were analysed in each sample. The cursor lines indicates the level of fluorescence above which cells were taken to be positively-labelled.

Figure 7

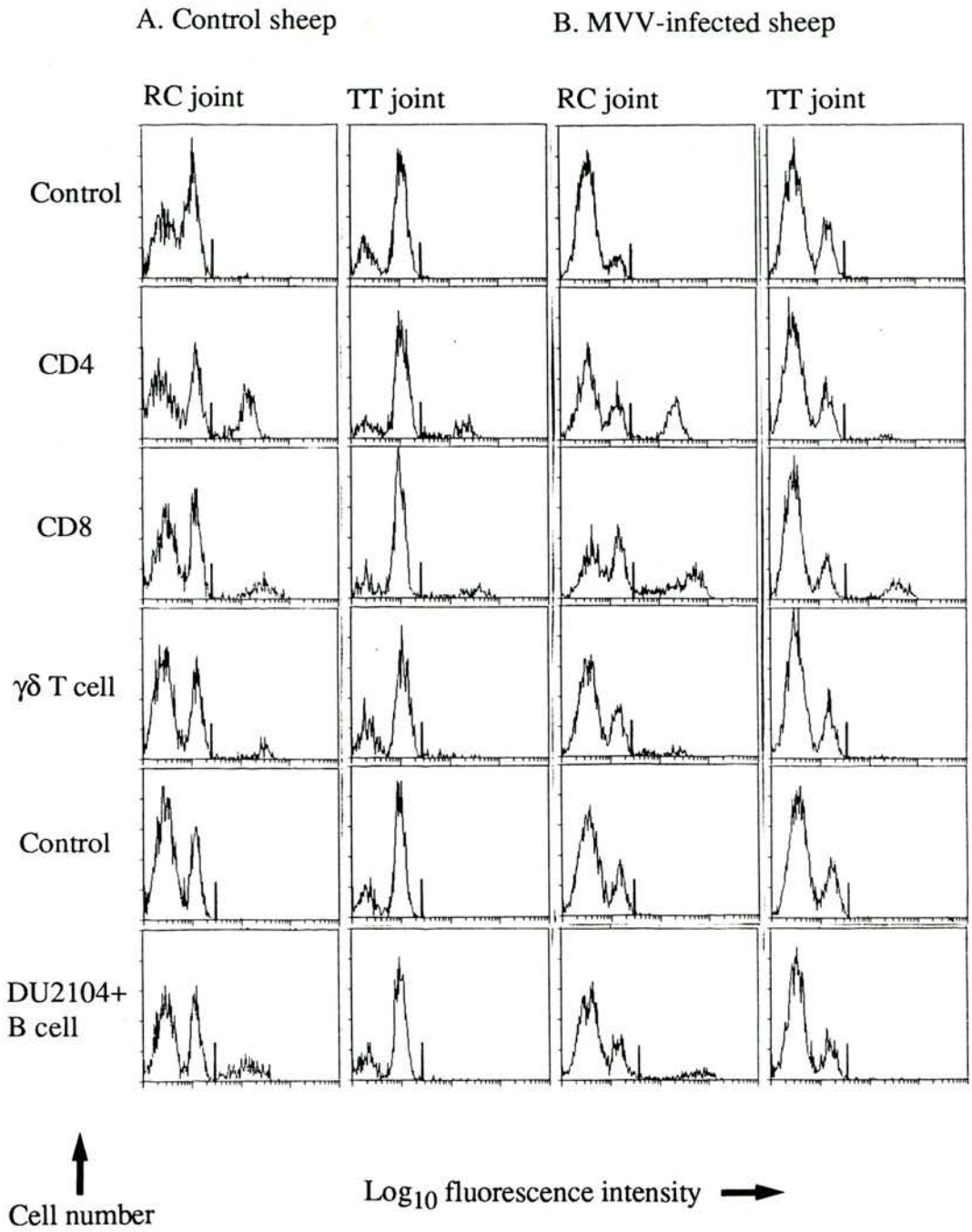

A. Control sheep
B. MVV-infected sheep
RC joint
TT joint
RC joint
TT joint
Control
CD4
CD8
γδ T cell
Control
DU2104+
B cell
Cell number
Log10 fluorescence intensity

Table 9. Lymphocyte populations in PB and SF from the RC and TT joints of MVV-infected and control sheep

			T cell			B cell			
			CD4 (%)	CD8 (%)	γδ (%)	(%)	CD4:CD8	αβ:γδ	T:B
MVV-infected	Clinically arthritic[a] (n=2)	1078 RC	16	63	5	7	0.4	17	13
		13 RC	16	76	1	5	0.2	92	19
		13 RC	24	72	3	8	0.2	29	12
	Non-clinically arthritic (n=9)	PB	31 (26-39)	20 (18-22)	4 (2-6)	29 (19-42)	1.6 (1.3-1.8)	12 (11-15)	1.8 (1.1-3.5)
		RC	21 (15-29)	65 (50-76)	5 (3-12)	5 (0-6)	0.3* (0.2-0.5)	14† (7-18)	19 (12-30)
		TT	23 (13-37)	60 (51-70)	6 (4-13)	5 (0-8)	0.4* (0.2-0.8)	13 (6-24)	16 (10-22)
Control (n=9)		PB	26 (23-30)	17 (12-20)	6 (5-10)	42 (34-50)	2 (1.4-2.3)	7 (4-10)	1.5 (1.1-1.8)
		RC	40 (30-42)	38 (30-44)	10 (6-28)	14 (2-22)	1.1 (0.8-1.7)	5 (1.6-11)	6 (3-36)
		TT	38 (31-43)	37 (27-55)	14 (4-17)	7 (4-22)	1.1 (0.6-1.6)	7 (5-21)	13 (4-28)

The figures for non-clinically arthritic and control sheep represent the median (upper value) and interquartile range (in parentheses). [a] The results of analysis of SF from 2 clinically arthritic sheep (1078 and 13) are shown. For sheep 13, SF from both RC joints was evaluated. (*$p<0.001$ compared to values for these joints from control sheep, †$p<0.01$ compared to values for this joint in control sheep)

the same joints from control sheep. The αβ:γδ T lymphocyte ratio was significantly higher in SF from the RC joints of MVV-infected sheep compared to controls ($p<0.01$).

The mean TNCC ± s.d. of SF from the RC and TT joints of these MVV-infected sheep were 1.7 ± 0.9 and 1.7 ± 2.4 respectively, as compared to 1.5 ± 1 and 1.1 ± 0.8 from the same joints from control sheep. These differences were not statistically significant and were comparable to previous values obtained from non-clinically arthritic MVV-infected and control sheep.

6.2.3. Clinically arthritic MVV-infected sheep

SF from 3 clinically arthritic joints (all RC joints) of 2 sheep (Numbers 1078 and 13) were evaluated. The percentage values of the lymphocyte subsets and the lymphocyte ratios for these joints are shown in Table 9.

Similar to non-clinically arthritic MVV-infected sheep, CD8+ T lymphocytes were the predominant T lymphocyte subset in SF from these sheep. These CD4:CD8 ratios in SF from arthritic joints were similar to those from non-clinically arthritic joints. However, the αβ:γδ T lymphocyte ratios were generally higher, suggesting a preferential infiltration of αβ T lymphocytes in the joints of these sheep.

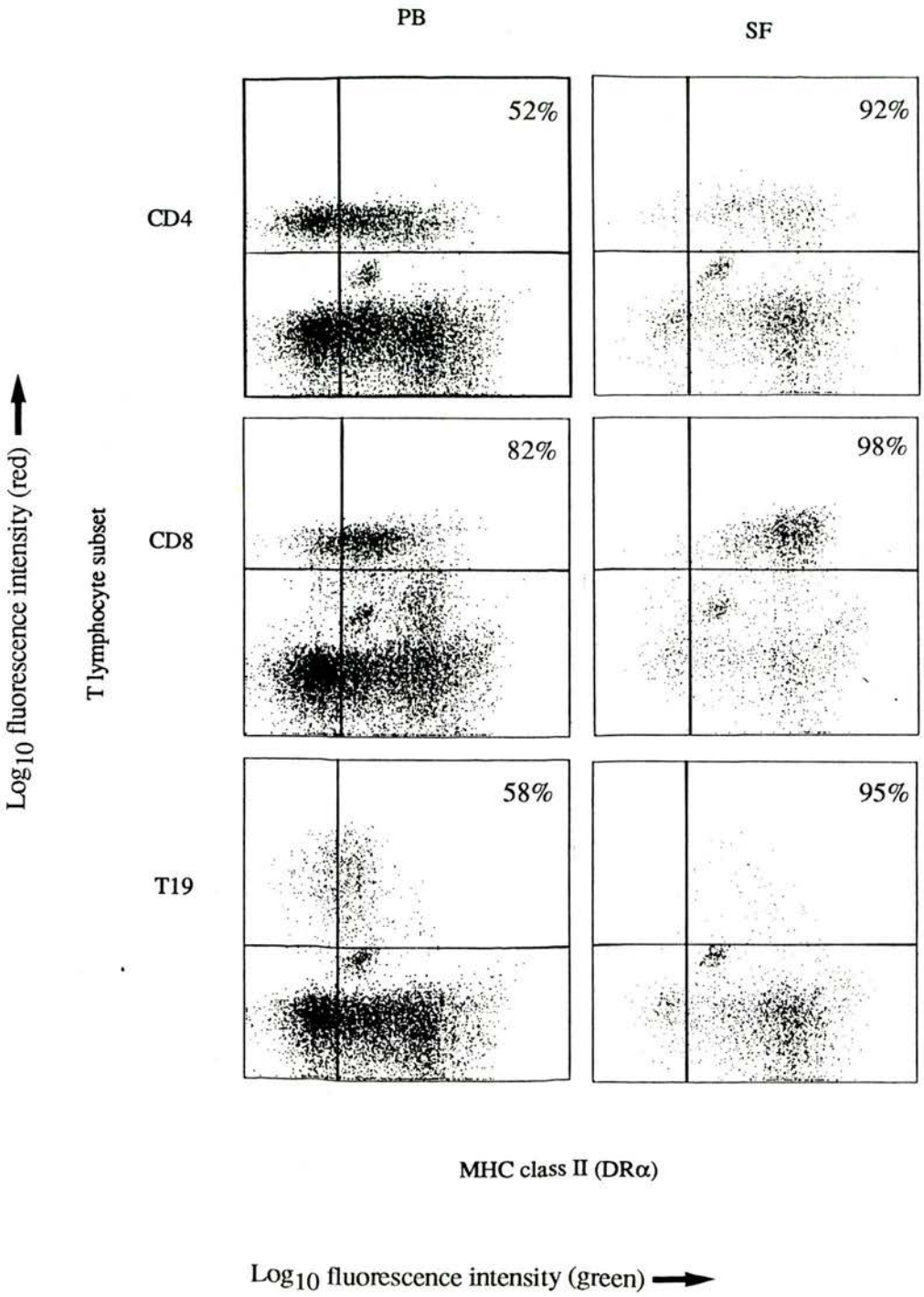
Additionally, for sheep 1078, the proportion of MHC class II-expressing SF T lymphocytes was determined by double-labelling analysis and compared to MHC class II expression by T lymphocytes from PB. This showed that the proportion of MHC class II-expressing T lymphocytes was higher in SF compared to paired PB (Figure 8).

The results of the above section suggested that there was a selective infiltration or retention of CD8+ T lymphocytes in SF in the RC and TT joints of non-clinically arthritic MVV-infected sheep compared to control sheep. The T:B lymphocyte ratio in SF from joints of arthritic and non-clinically arthritic sheep was high suggesting that B cells were either retained in the synovium, or infiltrate

Figure 8. MHC class II expression by T lymphocytes in PB and SF from a clinically arthritic MVV-infected sheep.

Briefly, single-cell suspensions from PB and SF were incubated in a mixture of Mab to T lymphocyte subsets (CD4+, CD8+ and T19+ γδ T cells) and MHC class II (DRα). Flow cytometric analysis of the lymphocyte populations was achieved using the live gates shown in Chapter 2, Figure 3.1.. 10,000 cells in PB and 2000 cells in SF were counted. The values shown in each graph are the proportion of MHC class II+ T lymphocytes.

Figure 8


PB
SF
52%
92%
CD4
82%
98%
CD8
58%
95%
T19
T lymphocyte subset
Log10 fluorescence intensity (red)
MHC class II (DRα)
Log10 fluorescence intensity (green)

normal or inflamed joints in small numbers. Although it was shown previously that moderate numbers of plasma cells were present in the synovia of some sheep, the paucity of DU2104+ B cells in synovia suggested that these cells infiltrate normal or inflamed joint tissues in smaller numbers than T lymphocytes.

7. Detection of MVV in the synovia from clinically arthritic MVV-infected sheep

Because of the limited quantities of synovia in the joints of arthritic and non-clinically arthritic MVV-infected sheep and the tissue requirements of other members of the Department, MVV isolation was attempted from synovia from the clinically arthritic joints of only 4 sheep (3 RC and a stifle joint from sheep 1550, 848, 13 and 80 respectively). MVV was isolated from these synovia using the methods described in Chapter 2, section 6.5. The presence of MVV was confirmed by immunofluorescence using Mab to the viral core protein P15 (Chapter 2, section 6.5).

Summary of clinicopathological findings

1. Clinical arthritis in MVV-infected sheep was uncommon. The most commonly affected joint was the carpal joint (uni- or bilaterally) with other joints occasionally affected.

2. Most clinically arthritic MVV-infected sheep had an erosive joint disease characterised by chronic inflammatory synovitis. SF TNCC were markedly raised in some joints and the predominant cell types in the SF from these sheep were lymphocytes and synoviocytes. CD8+ T lymphocytes were the predominant T lymphocyte in the synovia and SF of sheep with chronic synovitis. A small proportion of arthritic sheep had primarily non-inflammatory changes in their affected joints (probably degenerative or traumatic joint disease).

3. A proportion of non-clinically arthritic MVV-infected sheep had mild chronic inflammatory changes in their RC and TT joints as determined by synovial

histology and analysis of SF. Histological features including the presence of blood vessels with marked fibromuscular thickening of the intimal lining and infiltration with large numbers of LMN were observed in synovia from non-clinically arthritic MVV-infected sheep but not in tissues from control animals.

4. Immunocytochemistry of synovium and flow cytometric analysis of lymphocytes in SF revealed significant differences in the lymphocyte populations in both compartments in non-clinically arthritic MVV-infected sheep compared to controls. The CD4:CD8 T lymphocyte ratio was significantly lower in SF from the RC and TT joints of MVV-infected sheep, and increased numbers of CD8+ T cells were present in synovium from the RC joint of non-clinically arthritic MVV-infected sheep compared to these tissues from control sheep. Additionally, an increased proportion of cells in synovia from RC and TT joints of MVV-infected sheep expressed MHC class II antigens compared to synovia from the same joints of control sheep. Although a proportion of these MHC class II+ cells had a lymphoid morphology, the majority were LMN.

5. The presence of MVV was demonstrated in synovia from a minority of clinically arthritic joints from MVV-infected sheep by immunocytochemistry or by culture of synovial cells *in vitro*.

Discussion

The low incidence of clinical arthritis detected in this flock was in accordance with previous studies of sheep naturally-infected with MVV (Table 1). Most clinically arthritic sheep had erosive chronic inflammatory joint disease but severe erosions were uncommon, in accordance with the studies of CAEV-infected goats (Banks *et al* 1989). It is not known whether the development of severe erosive disease is due to additional cofactors or whether it is related to individual host genetic factors, in a similar fashion to that described for RA in humans where severe erosive disease is associated with particular MHC haplotypes (Nepom and Nepom 1993). The role of host genetic factors in the development of lentiviral

arthritis is poorly understood although certain MHC class I genes have been associated with resistance to develop arthritis in some breeds of CAEV-infected goats (Ruff and Lazary 1988).

No attempt was made in this study to isolate other infectious agents from the joints of MVV-infected sheep because of the limited quantities of synovium and SF present in arthritic joints and the demands of the procedures described in this chapter and others that were performed in parallel (Dr J. Cattermole, personal communication). Previous attempts to isolate bacteria, Chlamydia sps and Mycoplasma sps from clinically arthritic joints of MVV-infected sheep and CAEV-infected goats have been unsuccessful (Crawford *et al* 1980, Crawford and Adams 1981, Woodard *et al* 1982, Cutlip *et al* 1985a,b), although this does not exclude a role for these agents in the development of joint disease in these animals.

The histological appearance of synovium from clinically arthritic sheep with chronic inflammatory synovitis was similar to that described in previous reports of ruminant lentiviral arthritis, with the exception that large germinal centre-like structures were not seen (Table 1 and 2). However, one feature not previously reported were the blood vessels with marked intimal fibromuscular hyperplasia. These were observed in synovia from arthritic and non-arthritic sheep and closely resembled blood vessels that have been observed in synovia from humans infected with HIV (Dalton *et al* 1990) and from the parenchymal organs of children infected with this virus (Joshi *et al* 1987). The reason for the development of blood vessels with this appearance is not known although it has been suggested that local hypoxia may play a role (Dalton *et al* 1990). These blood vessels in this study were usually surrounded by large numbers of activated LMN. Cytokines such as PDGF (Raines *et al* 1989) from these cells could play an important role in their development. Alternatively, it is possible that they develop as a sequel to infection of endothelial cells with the virus. Although these cells are not believed to be capable of supporting productive viral infection (Gendelman *et al* 1985), endothelial cells harbouring viral mRNA have been demonstrated in synovium by

in situ hybridisation (Zink *et al* 1990). Because very few descriptions of ovine synovium have appeared in the literature it is not possible to say with certainty that they are a feature exclusive to MVV-associated arthritis. However, blood vessels with this appearance were not observed in synovia from the joints of sheep with AIA (Chapter 6), even in those sheep with long-standing chronic synovitis.

The predominance of CD8+ T lymphocytes in synovia and SF from clinically arthritic sheep is in accordance with previous observations of clinically arthritc MVV-infected sheep and CAEV-infected goats (Kennedy-Stoskopf *et al* 1989, Wilkinson *et al* 1992). A predominance of these lymphocytes has also been observed in synovia from humans with inflammatory arthropathies associated with HIV infection (Espinoza *et al* 1990) and Macaques infected with SIV (Roberts *et al* 1991). Thus, it would appear that this is a feature of arthropathies associated with lentiviral infections. CD8+ T lymphocytes also predominated in synovial tissues from non-clinically arthritic MVV-infected sheep. However, this subset also predominated in synovia from the TT joints of control sheep. Therefore, the significance of the findings from MVV-infected sheep are unclear. *In vitro* culture of monocytes from MVV-infected sheep results in up to 40% of these cells expressing low levels of CD8 antigen (Lee 1994b). If CD8-expressing monocytes or macrophages were included in the analysis of lymphocyte populations this could have resulted in abnormally high CD8+ lymphocyte counts from these tissues. This could have been resolved by double-labelling procedures.

The function of CD8+ T cells in ruminant lentiviral disease remains unclear. Cytotoxic CD8+ T cells have been demonstrated in the blood and efferent lymph of MVV-infected sheep (Bird *et al* 1993, Lee *et al* 1994a) but the functions of these cells in tissues has not been investigated. In addition to performing a cytotoxic function, CD8+ T cells have also been shown to suppress viral infection in HIV and SIV by the release of an uncharacterised lymphokine (Levy 1993, Ennen *et al* 1994), and in the presence of IL4 develop a B lymphocyte helper function (Erard *et al* 1993). Similarly, the function of CD4+ and γδ T cells in ruminant lentiviral

disease is not known although, *in vitro,* CD4+ T cells from PB can proliferate to viral antigens (Reyburn *et al* 1992) and are necessary to provide help for cytotoxic T cells (Stuhler and Walden 1993). Ovine γδ T lymphocytes have also been shown to be capable of performing a cytotoxic function *in vitro* (MacKay *et al* 1989).

Very large numbers of MHC class II-expressing cells were present in the synovia from clinically arthritic sheep. Although the identity of these cells was not confirmed, large numbers were clearly non-lymphoid. Whether these cells were macrophages, fibroblasts or DC could have been further investigated by double-labelling procedures. Although LV-IFN has been implicated as the cause of MHC class II expression in some tissues of experimentally-infected sheep (Kennedy *et al* 1985), it is unclear whether it is produced in the joints of arthritic sheep. Studies of human arthropathies have found very little evidence for γ-IFN production in joints (Firestein and Zwaifler 1987) and very few γ-IFN+ cells have been identified by *in situ* hybridisation in synovium from arthritic CAEV-infected goats (Lechner *et al* 1994). However, this latter study did show high levels of IL1β and TNFα expression in synovia from arthritic CAEV-infected goats. These cytokines have the potential to stimulate GM-CSF production (Alvaro-Garcia *et al* 1991) which is thought to play an important role in upregulating MHC class II expression in the joints of humans with RA (Xu *et al* 1989). IL1β could also play an important role in inducing the loss of proteoglycan (Pettipher *et al* 1989) from cartilage in the joints of these sheep (Harkiss *et al* 1995a).

Of particular interest was the observation that an increased proportion of cells in the SI of synovia from the RC and TT joints of non-clinically arthritic sheep expressed MHC class II compared to tissues from these joints of control sheep. Morphologically, most of these cells appeared to be LMN. It is likely that a proportion of these cells were monocyte-derived which raises the question of whether these cells were activated *in situ* or were pre-activated, prior to entry into the synovium. Very little is known regarding the activation status or requirements of monocytes in MVV-infected sheep or whether they display functional

differences with monocytes from non-infected sheep. The reasons for this elevated MHC class II expression are not clear. Small numbers of cells infected with the virus have recently been identified by *in situ* hybridisation in histologically normal synovium of MVV-infected sheep (Brodie *et al* 1995). No P15-expressing cells were identified in these tissues, in accordance with the results of this Chapter. Mechanisms do exist whereby cells latently-infected with these viruses could dysregulate local immune functions. Early viral gene products such as tat have been implicated in the pathogenesis of lesions in MVV and HIV infection (Buonaguro *et al* 1992, Neuveut *et al* 1993). *In vitro* tat has been shown to upregulate a variety of cellular genes including those encoding cytokines (Buonaguro *et al* 1992) and can act synergistically with cytokines in the development of some lesions eg Kaposi's sarcoma in HIV-infected individuals (Barillari *et al* 1993). Cells derived from human synovium infected *in vitro* with HTLV-1 produce GM-CSF (Sakai *et al* 1993). Abnormal production of this or other cytokines could result in upregulated MHC class II expression in synovial tissues *in vivo*. The functional consequences of increased numbers of MHC class II-expressing cells in the synovial tissues of these sheep are unclear. Given the role of MHC class II in antigen presentation and that the antigen-presenting capacity of a cell is related to the intensity of its expression (Unanue 1984), increased numbers of these cells in tissues may increase the responsiveness of the tissue to immune (Banks *et al* 1989) and non-immune stimuli, or to the development of autoimmune responses (Bottazzo *et al* 1983).

Increased numbers of CD1-expressing cells were present in some tissues from MVV-infected sheep. Although the function of CD1 is unclear, it can act as a ligand for some CD4- CD8- T lymphocytes (Porcelli *et al* 1989) and has recently been shown to act as a presentational molecule for lipid (Beckman *et al* 1994). High levels of CD1 expression are found on DC in AL and LC in skin and lower levels have been detected on peripheral blood monocytes and macrophages in some tissues (MacKay *et al* 1985). The factors responsible for modulation of CD1

expression in sheep are not known although *in vitro* studies of human monocytes have shown that GM-CSF can upregulate its expression (Kasinerk *et al* 1993). The intense expression of this antigen by some cells in synovia suggested they may have been DC.

Cells expressing the viral core protein P15 were detected in very small numbers in synovia from a minority of clinically arthritic MVV-infected sheep. Previous studies of experimental CAEV-infection in goats showed that viral protein was not detectable in synovium (by immunofluorescence) beyond day 9 post-infection and that virus could not always be isolated from the joints of chronically arthritic goats (Klevyer-Anderson *et al* 1984). Similarly, small numbers of viral protein-expressing cells were detected in the lungs of sheep naturally-infected with MVV, even when advanced pathological changes were present (Watt *et al* 1992b, Lujan *et al* 1994). It is possible that conventional immunocytochemical techniques are insufficiently sensitive to detect cells expressing low levels of viral protein. Alternatively, these cells may be elliminated by effector cells of the immune system.

Although there are many features of lentiviral arthritis that are poorly understood, an area of investigation that would be relevant to the understanding of this and other inflammatory arthropathies, that was not apparant from the studies described in this Chapter, is the characteristics of cell trafficking through inflamed joints. Investigating this phenomenon would rely upon cannulating afferent lymphatic vessels draining an inflamed joint. Given the low incidence of clinical arthritis in MVV-infected sheep, the slow, insidious clinical course of the disease, and the inconsistency with which inflammatory joint disease can be induced with this virus in older sheep (Dr. G. Harkiss, personal communication), it was concluded that an alternative model of chronic synovial inflammation, based on AIA in the rabbit, would be more appropriate to achieve this aim. Some authors have suggested that there may be some similarities in the pathogenesis of lentiviral arthritis and AIA (Harkiss 1994) and the characterisation of the latter disease in

sheep would provide useful comparative information for the results described in this Chapter. The generation and characterisation of AIA in sheep are described in the following Chapter.

CHAPTER 5

Characterisation of antigen-induced arthritis in sheep

Introduction

The ideal model of inflammatory arthritis with which to study cell trafficking through inflamed joints should have a number of characteristics. It should be easy to generate and the synovial tissues should have a predictable histological and immunopathological appearance at defined time points following induction to allow correlation with any changes that occur in the draining lymph. Also, the inflammation generated should be of sufficient severity to ensure that if there are changes in the composition of the draining lymph that these are sufficiently great to be detectable.

Numerous animal models of chronic inflammatory arthritis have been described in the literature (reviewed in Greenwald and Diamond 1988). The vast majority of these involve the use of laboratory rodents or rabbits as the host species although large animal models of inflammatory arthritis have also been described (Thorpe *et al* 1992). AIA was first generated in the rabbit by immunisation to autologous or heterologous fibrin followed by intraarticular injection of the immunising antigen (Dumonde and Glynn 1962). Since its original description, AIA has been generated in a number of species to investigate many different aspects of chronic inflammatory joint disease (reviewed in Chapter 1). Although chronic synovitis is a feature of the disease process, the mechanisms underlying its pathogenesis are poorly understood. Relatively few immunopathological studies have been performed to characterise the inflammatory infiltrate in the joints of animals with AIA. These studies have been performed on synovial tissues from rats up to seven days following induction of arthritis (Dijkstra *et al* 1987, Verschure *et al* 1989) and although performed on tissues from the acute stages of arthritis in the rabbit, the precise time at which these tissues were obtained following induction of arthritis was not stated (Wilkinson, J. *et al* 1993).

The antigen specificity of the lymphocytic infiltrate has been investigated only from the point of view of local antigen-specific antibody production. In this respect, up to 40% of synovial antibody production at 6 weeks post-induction of

AIA has been shown to be specific for the antigen inducing the arthritis (Cooke and Jasin 1972). Although the presence of antigen-specific T lymphocytes may be inferred from this finding, their presence within the joint has not been demonstrated.

The suitability of small ruminants as a host species for AIA has previously been shown by Banks *et al* (1987) who generated AIA in goats as part of a study to investigate the role of CAEV in joint inflammation. The only other form of inflammatory arthritis that has been generated in sheep, not involving the use of infectious agents, is CICIA (Thorpe *et al* 1992). However, AIA has an advantage over CICIA in terms of ease of antigen preparation. The characterisation of AIA in sheep would therefore provide useful background information prior to performing cannulation studies and comparative information for MVV-associated arthritis.

The aims of the following experiments were to characterise the inflammatory infiltrate in the joint at different stages during the development of AIA and investigate the antigen specificity of the lymphoid infiltrate in the joint.

Outline of methods

A group of adult Scottish Blackface ewes (n=24) were immunised to OVA in CFA. Two to three weeks later, AIA was generated by injecting 0.5mg OVA in to the right RC joint. The left RC joint was injected with 0.5ml sterile saline. This dose of antigen is at the lower end of the range usually used in rabbits (2-5mg/joint)(Cooke 1988) but has been shown in rabbits to be sufficient to result in a chronic synovitis of at least 8 weeks duration (Consden *et al* 1970) and was chosen because the aim was to generate a chronic synovitis and not a severe erosive arthritis. Because of financial limitations the same sheep would also be required for cannulation experiments described in Chapter 6.

The severity of joint enlargement was determined from calculation of the C:MC ratios at various time points following induction of arthritis (Chapter 2, section 2). Biopsies of synovium and aspirates of SF were obtained from both

OVA- and saline-challenged joints on days 1, 3, 16 and 30 following induction of arthritis. The limited number of time points were chosen to allow statistical analysis to be performed on the results and because afferent lymphatic cannulation procedures do not usually flow for longer than about 30 days. A portion of the synovial biopsy was fixed in 10% buffered formalin and processed for routine histological examination (H+E), and the remainder was snap frozen. Immunocytochemistry using Mab to B (DU2104+) and T lymphocyte subsets (CD4, CD8, γδ T lymphocyte), macrophages (CD14), MHC class II (DRα chain) and CD1 was performed on sections of frozen tissue. The proportions and densities of the T lymphocyte subsets were determined for synovium from sheep at days 3, 16 and 30 (Chapter 2, section 6.4.3.1.). Routine analysis of SF was performed to determine TNCC and differential cell counts and flow cytometry was used to characterise the B and T lymphocytic infiltrate. These results were compared with studies of PB performed in parallel.

The antigenic specificity of the B and T lymphocyte responses was determined by ELISA, immunocytochemistry and T lymphocyte proliferation assays.

Statistical analysis was performed using non-parametric tests. Comparison of values from more than 2 time points were initially compared by ANOVA using the Kruskal-Wallis test. Further comparisons were made using the Mann Whitney U-test or Wilcoxon Signed Rank test where the data was paired.

Results

PART I.

Phenotypic characterisation of AIA

1. Clinical evaluation

Following injection of OVA into the right RC joint all animals became acutely lame within 24 hours. At this time the joints appeared hot and were painful to manipulation. The vast majority of animals were non-weightbearing on the right forelimb for the first 2 or 3 days after which time limb use gradually increased.

Most animals showed no signs of lameness 10 to 14 days following antigenic challenge. One animal remained markedly lame for 6 weeks and was euthanased and one remained almost non-weightbearing for 2 weeks and was euthanased on day 16. In both cases the periarticular soft tissue swelling was severe and histopathological examination of synovium from the affected joint of the latter animal showed the presence of large numbers of neutrophils. It was concluded that OVA had either been injected into the joint tissue as opposed to the joint space or that bacteria had been inadvertently innoculated with the antigen.

The C:MC ratios of the OVA- and saline-challenged joints are shown in Figure 1. The differences in paired ratios at each time point from day 1 to day 16 were statistically significant ($p<0.014$ at all time points). At day 30, 4 of 6 sheep had mild soft tissue thickening of the right RC joint compared to the left RC joint.

2. Synovial histology and immunohistology

The numbers of CD4, CD8 and γδ T lymphocytes were quantitated according to the methods described in Chapter 2, section 6.4. This was not performed on day 1 because tissues from only 2 sheep were evaluated. The expression of CD1, MHC class II (DRα chain) and CD14 were determined for synovia from the OVA-challenged joint of 4 sheep biopsied on days 3, 16 and 30 following induction of arthritis.

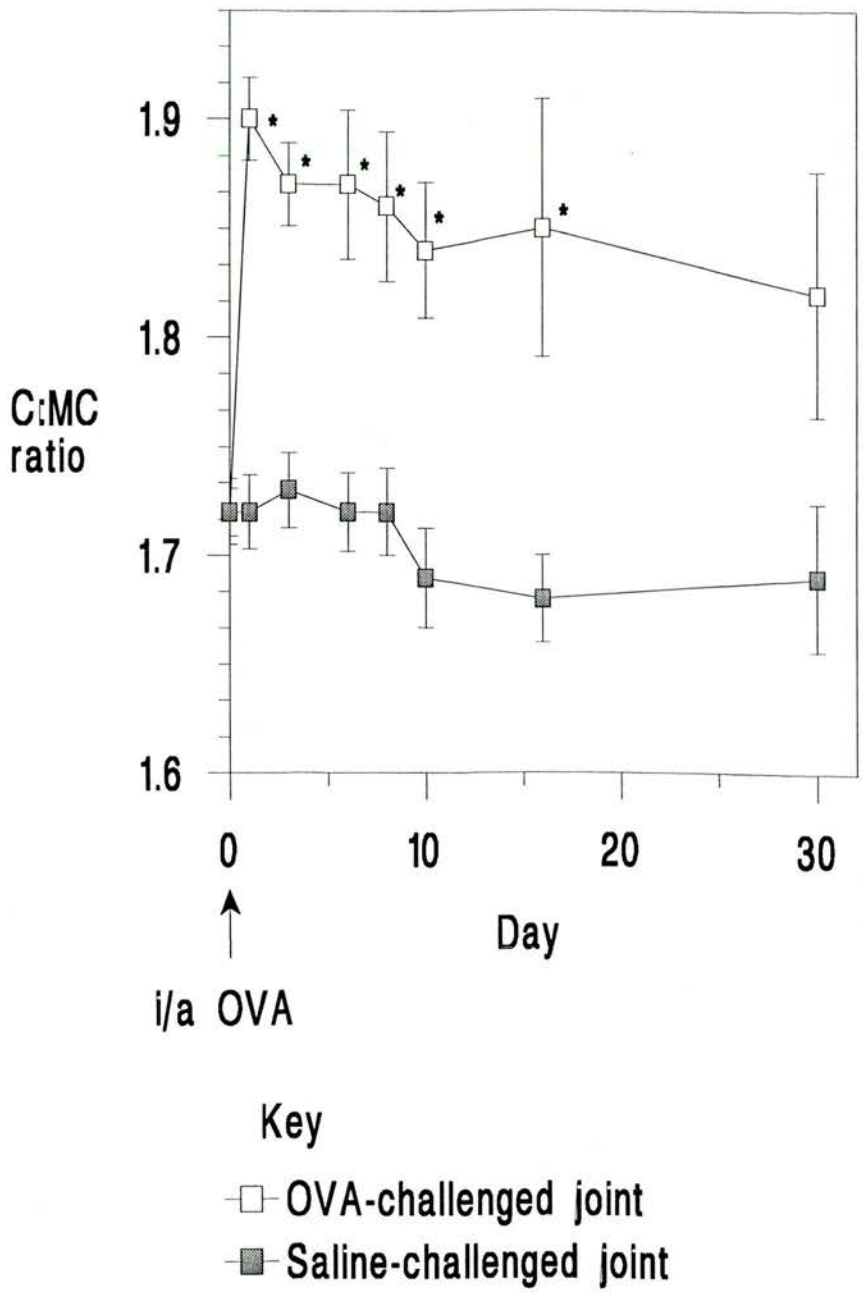
2.1. Day 1 post-induction of AIA (n=4)

2 sheep had synovial biopsy performed at this time point. The severity of the inflammatory response and associated haemorrhage prevented the acquisition of synovium and so the remaining 2 sheep were euthanased and synovium removed at post mortem. In both cases histological examination showed widespread extravasation of red blood cells and large numbers of neutrophils were diffusely scattered throughout the SI. Large areas of synovium appeared to be denuded of SLC and areas of fibrin deposition were adherent to the superficial surface of the

Figure 1. Carpal: Metacarpal (C:MC) ratios following the generation of AIA

C:MC ratios were determined from measurements of carpal and metacarpal circumferences made before and at various time points following injections of 0.5mg OVA into the right RC joint (open box) and 0.5ml sterile saline into the left RC joint (filled box). Measurements were made from 19 OVA-primed sheep prior to generation of AIA but were not made from sheep after biopsy of the synovial lining. Hence the numbers of sheep from which ratios were determined fell from this value to 6 by day 30. The values shown are the mean ± s.e.m.. Statistical analysis of paired RC joints confirmed that the OVA-challenged joint was significantly enlarged at all time points (*$p<0.008$) compared to the saline-challenged joint, except at day 30.

Figure 1


1.9
1.8
1.7
1.6
C:MC ratio
0
10
20
30
Day
i/a OVA
Key
OVA-challenged joint
Saline-challenged joint

SI (confirmed in several cases with Masson's Trichrome histological stain). Large numbers of blood vessels had swollen vacuolated endothelial linings that frequently obliterated the lumen of the vessel whilst others were filled with a homogenous eosinophilic proteinaceous debris. The widespread tissue disruption made identification of individual cell types more difficult but moderate numbers of lymphoid cells did appear to be present.

Immunohistological examination of synovium from the right RC joints from these 2 sheep showed small to moderate numbers of scattered lymphoid cells. In one tissue the numbers of CD8+ T lymphocytes exceeded the number of CD4+ T lymphocytes and in the other, the numbers were approximately equal. In both cases these cells exceeded the numbers of γδ T cells. Very few DU2104+ B lymphocytes were observed.

In contrast, synovium from the left RC joint showed more minor changes with small to moderate numbers of neutrophils present in the the more superficial areas of SI and some blood vessel lumena were filled with homogenous eosinophilic proteinaceous debris. Small numbers of all 3 T lymphocyte subsets but no DU2104+ B cells were present in the synovial lining of one joint whilst the other had only small numbers of CD8+ T lymphocytes. In both cases the number of T lymphocytes in the right RC joint greatly exceeded the numbers in the left RC joint.

2.2. Day 3 post-induction of AIA (n=7)

Synovium from the right RC joint showed mild to moderate SLC hyperplasia in 5 of 7 tissues. The most superficial SLC in all cases had abundant vacuolated cytoplasm. In all tissues large numbers of congested blood vessels were seen in the SI. Occasional to moderate numbers of neutrophils were present in the more superficial areas of the SI in all tissues. Large numbers of extravasated red blood cells were present in the SI and areas of fibrin were adherent to the superficial SLC in all tissues. Large numbers of lymphoid cells were diffusely scattered throughout the SI although loosely organised focal and perivascular aggregates were seen in 5

of 7 tissues.The number of plasma cells appeared to be small relative to the number of small lymphocytes. Large numbers of LMN were also present.

Immunohistological examination showed that the predominant T cell subset was CD4+ (Figure 2 and 3). The vast majority of these cells were diffusely scattered throughout the SI but in 5 of 7 tissues small numbers (2 to 6) of focal aggregates were seen. ANOVA confirmed that the CD4:CD8 and αβ:γδ T lymphocyte ratios varied significantly on days 3, 16 and 30 ($p<0.013$ and 0.014 for the CD4:CD8 and αβ:γδ T cell ratios respectively). The CD4:CD8 ratio on day 3 (median of 3.7) was significantly higher than on days 16 and 30 (median of 1.4 and 1 respectively)($p<0.009$), and compared to paired synovium from the left RC joint ($p<0.04$). The number of γδ T cells approximated to the number of CD8+ T cells (Figure 2 and 3). Most of these cells were diffusely scattered throughout the SI but small numbers were present within the lymphoid aggregates. The αβ:γδ T lymphocyte ratio was significantly lower on day 3 (median of 5.5) than on days 16 and 30 (median of 11 and 21 respectively)($p<0.03$ and 0.009 for these days respectively). The number of DU2104+ B cells was small in all tissues except one where quite large numbers of weakly positively-stained cells were found diffusely scattered throughout the SI (Figure 4). The quality of immunostaining obtained with Mab to MHC class II and CD14 precluded accurate assessment of staining with these reagents. However, moderate numbers of CD1-expressing cells in the SI and lower levels of the SLC layer were present in several sections (Figure 4).

By comparison, the changes in synovium from the left RC joint were minimal. Congestion of the superficial blood vessels was universally present but relatively few neutrophils and extravasated erythrocytes were present in the SI. Small numbers of scattered lymphoid cells were seen in 6 of 7 tissues. Although small in number, CD4+ T lymphocytes predominated over CD8+ T lymphocytes. ANOVA confirmed that the CD4:CD8 but not the αβ:γδ T lymphocyte ratio varied significantly on days 3, 16 and 30 ($p<0.03$). The CD4:CD8 ratio (median of 1.1) was significantly raised compared to that from day 30 (median of 0.4)($p<0.05$) but

Figure 2. T lymphocyte subsets in the synovial lining of sheep with AIA

Frozen sections of synovium from day 3 (a, c, e) and day 30 (b, d, f) following the generation of AIA were labelled with Mab to CD4+ (a, b), CD8+ (c, d) and γδ T (e, f) lymphocytes. (Immunoperoxidase technique, original magnification x156).

Figure 2

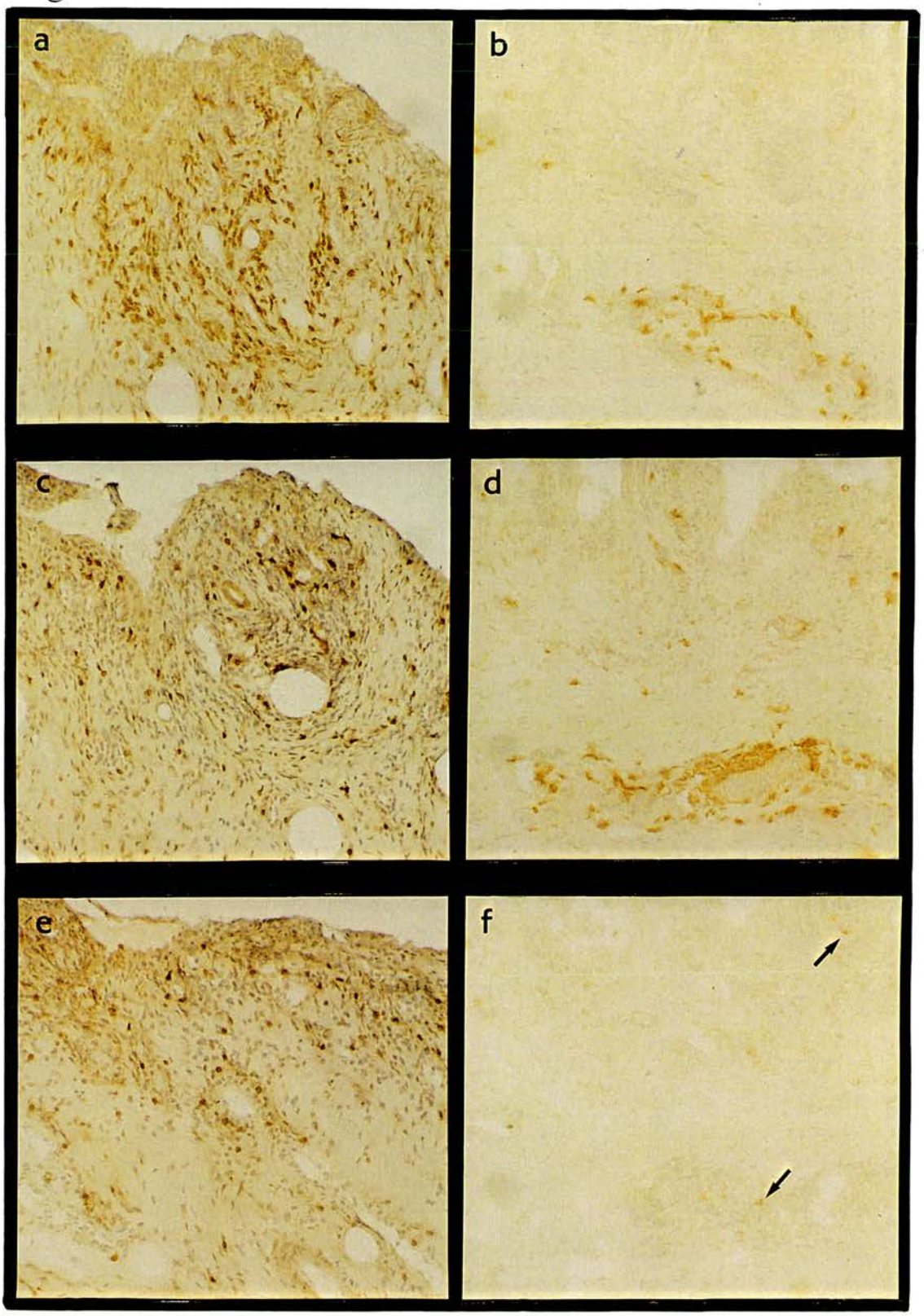


Figure 3. T lymphocyte densities in the synovial lining from sheep with AIA (OVA-challenged joint).

The densities of CD4+, CD8+ and γδ T lymphocytes in the synovial lining of sheep biopsied on days 3, 16 and 30 (n=7, 7 and 6 for these time points respectively) following the generation of AIA were determined from frozen sections of synovium stained using an immunoperoxidase technique. T lymphocyte densities were determined from identical areas of sequential sections stained by this technique. The bars show the median values for each subset at each time point.
(*$p<0.05$ and **$p<0.005$ compared to the values obtained on day 30)

Figure 3

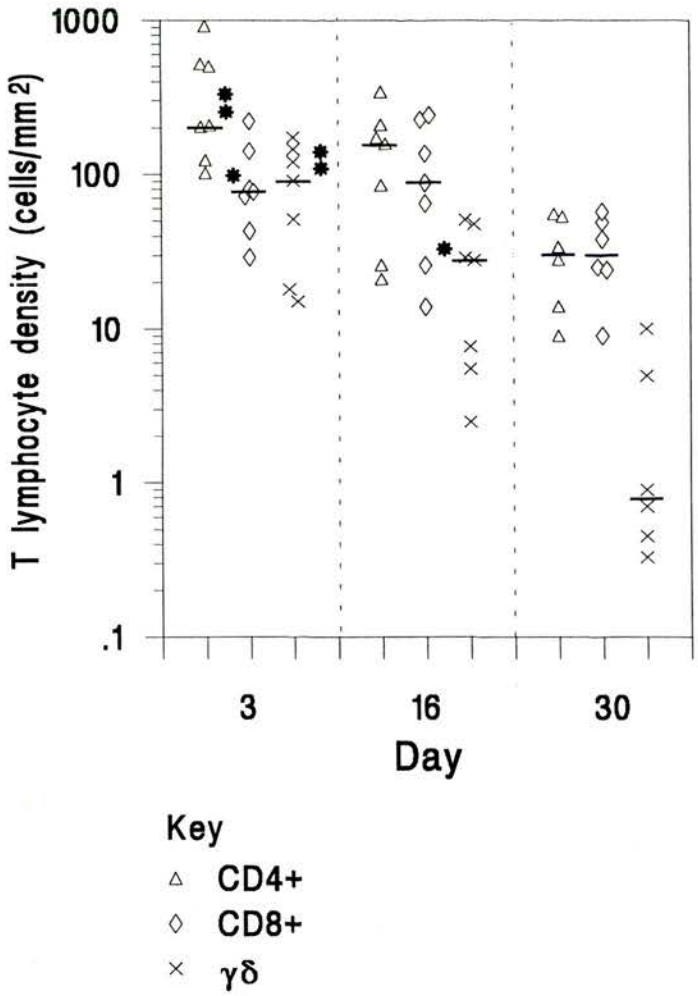
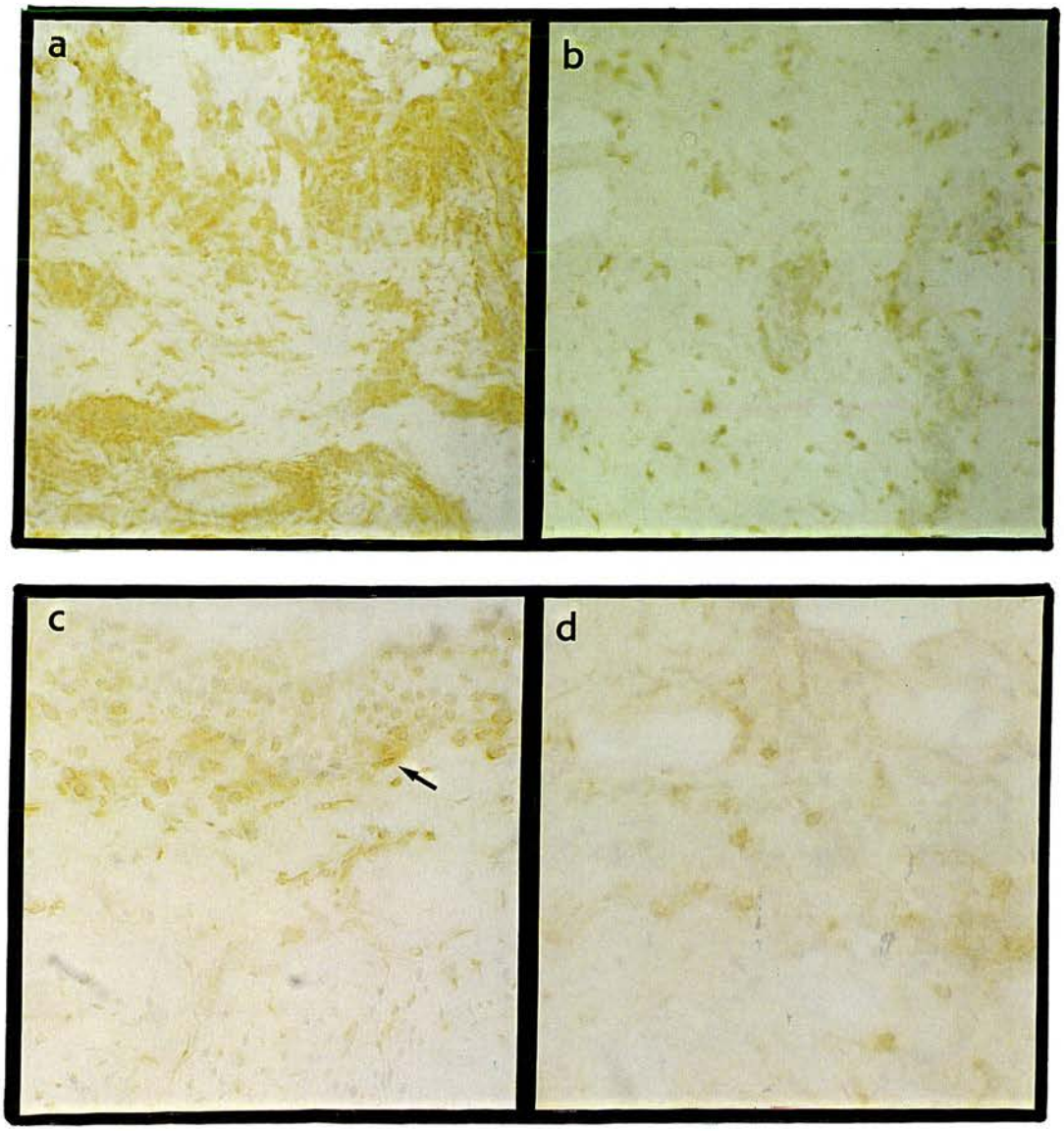

1000
100
10
1
.1
T lymphocyte density (cells/mm2)
3
16
30
Day
Key
CD4+
CD8+
γδ

Figure 4. Identification of cells in synovium expressing MHC class II, CD14, CD1 and DU2104 antigens

Frozen sections of synovium biopsied on day 16 following generation of AIA were labelled with Mab to MHC class II (a) and CD14 (b)(Immunoperoxidase technique, original magnification x156). Large numbers of MHC class II-expressing cells are visible in the SLC and SI layers in (a) and moderate numbers of CD14+ macrophages are present in the SI in (b). Frozen sections of synovium biopsied on day 3 were labelled with Mab to CD1 (c) and DU2104 (d)(original magnification x390). The arrow in (c) indicates a weakly-stained cell at the base of the SLC layer and small numbers of weakly-stained DU2104+ B cells are present in the SI in (d).

Figure 4



not compared to day 16 (median of 0.9). Small numbers of diffusely scattered DU2104+ B cells were present in a minority of tissues.

2.3. Day 16 post-induction of AIA (n=7)

Mild to moderate SLC hyperplasia was present in 5 of 7 tissues from the right RC joint. Large numbers of lymphoid cells were seen in all tissues and only occasional neutrophils were present in the SI (Figure 5). Plasma cells represented the bulk of the lymphoid infiltrate in some tissues whilst small lymphocytes predominated in others. Although moderate numbers of these cells were diffusely scattered throughout the SI, more clearly defined focal and perivascular aggregates were present compared to day 3. Fibrosis was extensive with very little adipose tissue present in any of the tissues.

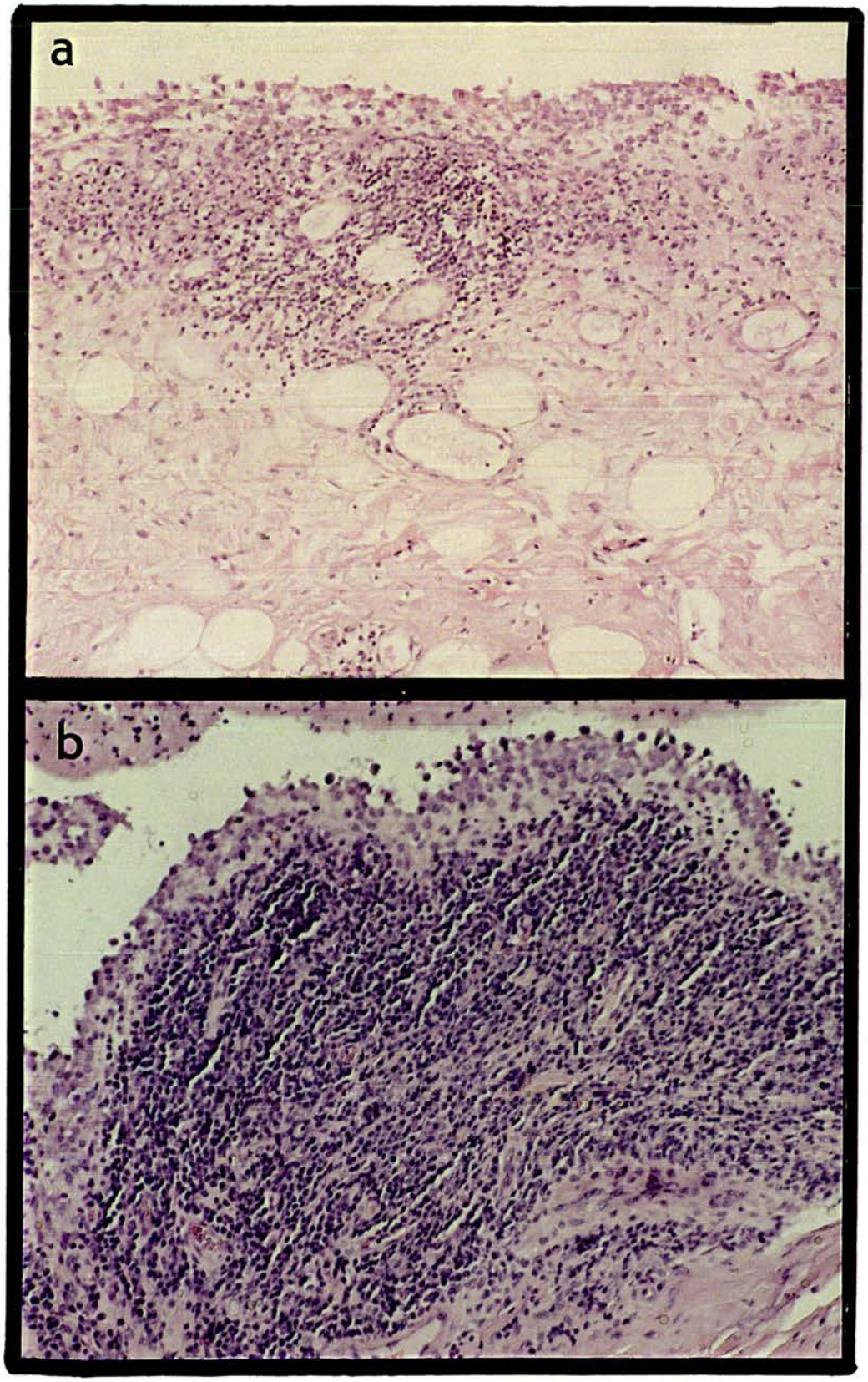
In 5 of 7 tissues the number of CD4+ T cells exceeded the number of CD8+ T cells (The densities of the T lymphocyte subsets are shown in Figure 3). Large numbers of CD4+ T cells were present in focal and perivascular aggregates immediately beneath the SLC, as well as diffusely scattered throughout the SI. CD8+ T cells were present in these lymphoid aggregates but always in smaller numbers compared to the CD4+ T cells. However, more CD8+ T cells were present in the interfollicular areas and immediately beneath the SLC. Small numbers of γδ T lymphocytes were diffusely scattered throughout the SI and were only rarely seen within the lymphoid follicle-like structures. Very small numbers of DU2104+ B cells were present in 3 of 7 tissues. Moderate numbers of CD14+ macrophages were present and a large proportion of SLC and cells in the SI were MHC class II+ (Figure 4). Only occasional CD1-expressing cells were observed in the SI.

Synovium from the left RC joint resembled tissue from previously described control sheep. Occasional neutrophils and lymphoid cells were seen but no tissues showed evidence of SLC hyperplasia. CD8+ T cells exceeded CD4+ T cells in 5 of 7 tissues and no DU2104+ B cells were observed.

Figure 5. Histological appearance of synovium from AIA (a) and following a flare reaction (b)(H+E, original magnification x156).

(a) Synovium biopsied at day 16 following generation of AIA. There is a mild degree of thickening of the SLC layer and an aggregate of lymphoid cells immediately beneath this layer. Increased numbers of LMN cells are present in the SI and much of the adipose tissue in this layer has been replaced by fibrous tissue.
(b) Synovium from a sheep 16 days after induction of a flare response. There is moderate thickening of the SLC layer and very large numbers of lymphoid cells in the SI.

Figure 5



2.4. Day 30 post-induction of AIA (n=6)

Synovium from the right RC joint from all sheep showed evidence of chronic inflammatory synovitis. There appeared to be little difference in the severity of the chronic inflammatory change comparing those sheep with joint enlargement (n=4) and those without (n=2). Moderate SLC hyperplasia was present in 3 of 6 tissues and lymphoid infiltrates characterised by large focal and perivascular aggregates were present in 4 of 6 tissues. Similar to day 16 the number of plasma cells relative to small lymphocytes varied.

The CD4:CD8 T lymphocyte ratio was quite variable with 3 of 6 tissues having a predominance of CD4+ T cells and in the remainder, CD8+ T cells predominated (Figure 2). The distribution of these cells was very similar to day 16 with small numbers of focal and perivascular aggregates present in all tissues. Although the densities of all T cell subsets was less than at day 16, the reductions were statistically significant only for the γδ T cell subset ($p<0.02$)(Figure 2 and 3). However the densities of all T cell subsets were significantly reduced compared to day 3 ($p<0.03$). Only occasional weakly-stained DU2104+ B cells were present in 2 of 6 tissues. MHC class II and CD1 expression appeared similar to that present on day 16.

Synovium from the left RC joint had a very similar histological appearance to that obtained from this joint on day 16. In spite of their low numbers, CD8+ T cells always predominated over CD4+ T cells (absence of CD4+ T lymphocytes in 2 tissues precluded paired analysis of ratios). No DU2104+ B lymphocytes were present in any tissues. MHC class II and CD1 expression were similar to that observed on day 16.

2.5. Histology of synovium from sheep with AIA generated 9 to 14 months previously

Synovium was obtained from right and left RC joints of 5 sheep, 9 to 14 months following induction of AIA. All sheep had both joints biopsied up to 30

days following induction of arthritis. Synovium from the right RC joints of all sheep had chronic inflammatory changes characterised by mild SLC hyperplasia, fibrosis, increased numbers of LMN and by the presence of very large numbers of lymphoid cells in 4 of 5 tissues. Numerous focal and perivascular aggregates were present in these tissues and large numbers of plasma cells were present.

Histological changes in synovium from the left RC joint were minimal with the exception of mild SLC hyperplasia and widespread fibrosis in most cases. However, large numbers of lymphoid cells were present in synovium from 1 sheep.

No erosions of articular cartilage were present in the joints of any these sheep.

3. SF analysis

TNCC and differential cell counts were performed on all SF. The proportions of the B and T lymphocyte subsets in SF from right and left RC joints was determined by flow cytometry on day 3, 16 and 30 following induction of arthritis. From these results were calculated the CD4:CD8, $\alpha\beta$:$\gamma\delta$ and T:B lymphocyte ratios which were compared with results from paired synovial lining (n=6 at each time point). These ratios for lymphocytes in PB were determined for 4, 6 and 4 sheep on days 3, 16 and 30 respectively. The expression of MHC class II (pan MHC class II and DQα) and IL2r by the T lymphocyte subsets in SF from the right RC joint was determined by double-labelling and compared with results from paired PB. In addition, the expression of a range of CAM on CD2+ T lymphocytes in paired PB and SF was evaluated in a small number of sheep. Because of the paucity of lymphocytes in SF from the left RC joint, double-labelling was performed in only a limited number of sheep.

3.1 Total and differential cell counts

Table 1 shows the cellular composition of SF from the right and left RC joints at different time points following induction of AIA. For the right and left RC joints ANOVA confirmed that the TNCC varied significantly at the time points evaluated

Table 1. Differential cell counts of SF from sheep with AIA

	Joint[a]	TNCC ($x10^5$/ml)	Neutrophils (%)	Lymphocytes (%)	Synoviocytes (%)	Macrophages (%)
Day 1	RC	710 ± 255*	86 ± 16	1 ± 1.4	13 ± 15	0.5 ± 0.6
(n=4)		(340-913)	(61-96)	(0-3)	(3-36)	(0-1)
	LC	369 ± 64*	85 ± 3.5	2 ± 1.5	13 ± 3	-
		(300-432)	(80-88)	(0.5-4)	(10-16)	-
Day 3	RC	897 ± 1038*	78 ± 13	4.3 ± 1.7	12 ± 6	5.6 ± 7.9
(n=6)		(136-2940)	(56-94)	(2-6.5)	(3-20)	(0.5-20)
	LC	49 ± 38**	39 ± 17	4.6 ± 2.8	52 ± 23	5.1 ± 7.1
		(17-119)	(13-57)	(1.5-7.5)	(22-85)	(0-15)
Day 16	RC	63 ± 45	41 ± 29	16 ± 6	38 ± 30	5.4 ± 3.5
(n=7)		(9-140)	(7-70)	(7.5-2.4)	(12-83)	(1.5-12)
	LC	3.3 ± 2	17 ± 18	11 ± 4.4	66 ± 14	3.8 ± 2.8
		(1.1-7)	(0-42)	(6-18)	(49-84)	(0-8)
Day 30	RC	56 ± 51	35 ± 26	28 ± 7	34 ± 23	3.3 ± 3.4
(n=6)		(13-150)	(3-71)	(17-39)	(1.5-70)	(0.5-10)
	LC	2.6 ± 2	6 ± 9	10 ± 8	84 ± 15	2.3 ± 4.1
		(0.6-5.8)	(0.5-23)	(0-20)	(62-99)	(0-11)

Values shown are the mean ± s.d. with the range shown in parentheses below.

[a] The right RC joint (RC) was injected with 0.5mg OVA in 0.5ml of sterile saline and the left RC joint (LC) was injected with 0.5ml sterile saline.

For values obtained from the right RC joint *$p<0.02$ compared to the values obtained from this joint on day 16 and 30. For the left RC joint *$p<0.02$ compared to the values obtained on day 3 from this joint and **$p<0.005$ compared to the values obtained on day 16 and 30. On day 3, 16 and 30 the values obtained from the right RC joint were significantly elevated ($p<0.04$) compared to those from paired left RC joints.

($p<0.009$ and 0.001 for the right and left RC joints respectively). For the right RC joint the reduction in TNCC on day 16 and 30 compared to the earlier time points were statistically significant ($p<0.02$ for all comparisons). For the left RC joint the TNCC on day 1 was significantly higher than on day 3 ($p<0.01$) which, in turn, was significantly higher than at subsequent time points ($p<0.004$). The differences in TNCC comparing paired right and left RC joints were statistically significant on days 3, 16 and 30 ($p<0.04$ for all 3 time points).

In the OVA-challenged joint neutrophils predominated on days 1 and 3, whereas the proportions of lymphocyte and synoviocytes were increased at the later time points. In SF from the saline-challenged joint neutrophils predominated on day 1 but synoviocytes predominated from day 3 onwards.

3.2. Flow cytometric analysis of T lymphocytes in PB and SF

3.2.1. B and T lymphocyte subsets in PB and SF

The percentage values obtained for B and T lymphocyte subsets in SF are shown in Table 2. ANOVA showed that the CD4:CD8 T lymphocyte ratio in SF from the OVA- and saline-challenged joints varied significantly between the 3 time points ($p<0.013$ and <0.03 for the right and left RC joints respectively). The CD4:CD8 ratios in the right and left RC joints on day 3 were significantly higher than for the same joint on day 16 ($p<0.008$ and <0.02 for the right and left RC joints respectively) and day 30 for the right RC joint ($p<0.03$). These results were similar to those obtained from synovium. Paired comparison of the CD4:CD8 and $\alpha\beta$:$\gamma\delta$ T lymphocyte ratios from right and left RC joints revealed no statistically significant differences with the exception of the CD4:CD8 ratio on day 30 which was significantly higher in the OVA-challenged joint ($p<0.04$).

ANOVA of the $\alpha\beta$:$\gamma\delta$ T lymphocyte ratios for the OVA-challenged joint confirmed that these ratios varied significantly between the 3 time points ($p<0.014$). This ratio on day 30 was significantly higher than at the previous time points ($p<0.03$).

Table 2. Lymphocyte subsets in SF following generation of AIA

Day	Joint[a]	T cell			B cell	Lymphocyte ratios		
		CD4 (%)	CD8 (%)	γδ (%)	(%)	CD4:CD8	αβ:γδ	T:B
3	OVA	53 (40-63)	17 (13-19)	23 (16-32)	7 (6-9)	3.5* (2.4-6)	3.1 (2.6-12)	15 (13-37)
	Control	41 (36-47)	15 (15-30)	20 (15-36)	13 (6-24)	1.7* (1.3-4.3)	3.6 (1.5-5.8)	11 (3-37)
16	OVA	51 (44-53)	34 (28-36)	10 (5-15)	8 (6-11)	1.5 (1.3-1.8)	8.6 (5.5-15)	15 (11-37)
	Control	35 (29-46)	48 (37-50)	12 (9-17)	7 (5-10)	0.7 (0.6-1.3)	7.4 (4.6-9.8)	15 (10-22)
30	OVA	58 (49-64)	32 (26-43)	7 (6-9)	2 (1-3)	1.9 (1.2-2.5)	13† (10-16)	49† (37-99)
	Control	48 (38-52)	42 (29-47)	12 (8-17)	2 (0-5)	1.2 (0.8-1.8)	7.4 (4.5-9.8)	25 (13-32)

Values shown are the median (n=6 for the OVA-challenged and control joints at each time point except for values obtained for B cells where n=4) with the interquartile range shown in parentheses below.

For the OVA-challenged joint *$p<0.03$ compared to the later time points. For the control joint *$p<0.02$ compared to values from this joint obtained on day 16. †$p<0.03$ compared to the values obtained from the same joint on days 3 and 16.

The proportion of DU2104+ B lymphocytes in SF from both RC joints was evaluated in 4 sheep at each time point. The T:B ratio in SF from the right RC joint on day 30 was significantly raised compared to day 3 or 16 ($p<0.03$ for both time points) for the same joint. Flow cytometric analysis failed to reveal significant numbers of DU2104+ B lymphocytes in the left RC joint on day 30 .

3.2.2. Activation status of T lymphocytes in paired PB and SF

Paired PB and SF from the right RC joint was obtained from 4, 6 and 4 sheep on days 3, 16, and 30 respectively. A sufficient number of cells was recovered from the left RC joint from 2, 4 and 1 sheep on days 3, 16 and 30 respectively to allow evaluation of MHC class II (pan reagent) and IL2r expression on T lymphocytes in SF from this joint.

Typical scatter dot plots of paired PB and SF T lymphocytes are shown in Figure 6 and 7. For statistical analysis the results for each T lymphocyte subset for MHC class II and IL2r from the 3 time points were pooled. These results are shown in Figure 8.1 and 8.2.

MHC class II was expressed by a significantly higher proportion of all 3 T lymphocyte subsets in SF from the right RC joint compared to these subsets in paired PB ($p<0.003$ for all 3 subsets). Additionally, the percentage of CD8+ T lymphocytes expressing MHC class II was significantly higher than for CD4+ or $\gamma\delta$ T lymphocytes ($p<0.001$ and <0.006 for these subsets respectively). The level of expression of MHC class II was generally higher on SF lymphocytes than those in paired PB.

MHC class II expression by SF T lymphocytes from the left RC joint showed similar results with a significant increase in the proportion of positively-labelled CD4+ and CD8+ T lymphocytes ($p<0.02$ and <0.04 for these subsets respectively) compared to PB. Similarly to the right RC joint, significantly more CD8+ T lymphocytes expressed MHC class II compared to CD4+ T lymphocytes ($p<0.003$). Although the proportion of MHC class II-expressing $\gamma\delta$ T lymphocytes

Figure 6. Double-labelling flow cytometry scatter plots of T lymphocytes from PB and SF (OVA-challenged joint).

Equal concentrations of cells from PB and SF from the OVA-challenged joint of a sheep with AIA (sampled on day 16 following generation of AIA) were incubated in Mab to CD4, CD8 or T19 ($\gamma\delta$ T lymphocytes), and MHC class II [pan MHC class II (i) and DQ-specific (ii) Mab] or IL2r (iii). 10,000 cells were counted for each combination of Mab.

Figure 6

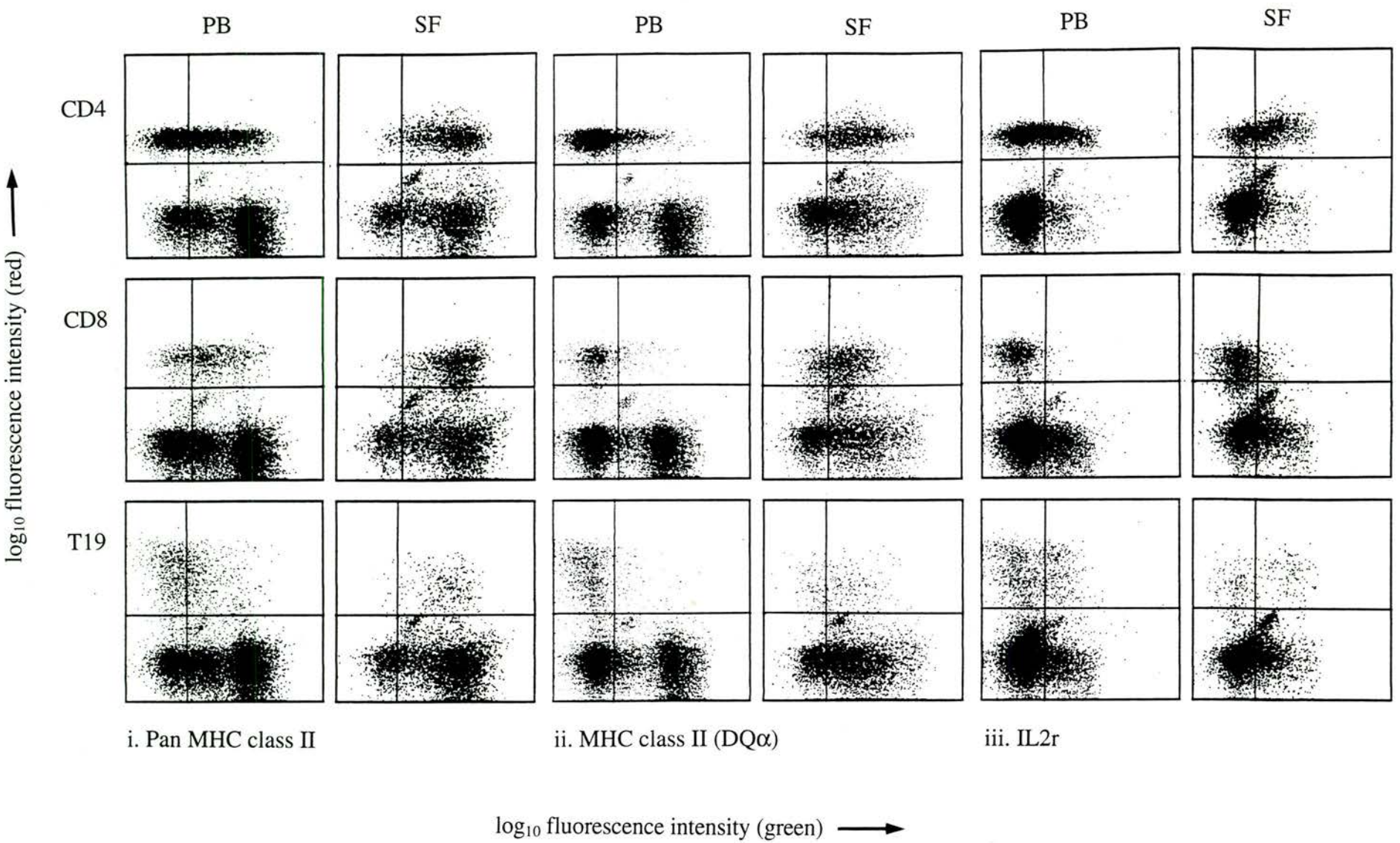

PB
SF
PB
SF
PB
SF
CD4
CD8
T19
log10 fluorescence intensity (red)
i. Pan MHC class II
ii. MHC class II (DQα)
iii. IL2r
log10 fluorescence intensity (green)

Figure 7. Double-labelling flow cytometry scatter plots of T lymphocytes from PB and SF (saline-challenged joint).

Cells in PB and SF from the saline-challenged joint of a sheep with AIA (day 16 following generation of AIA) were incubated in Mab to CD4, CD8 or T19, and MHC class II (i) or IL2r (ii). 10,000 lymphocytes were counted in PB and 1000 were counted in SF.

Figure 7

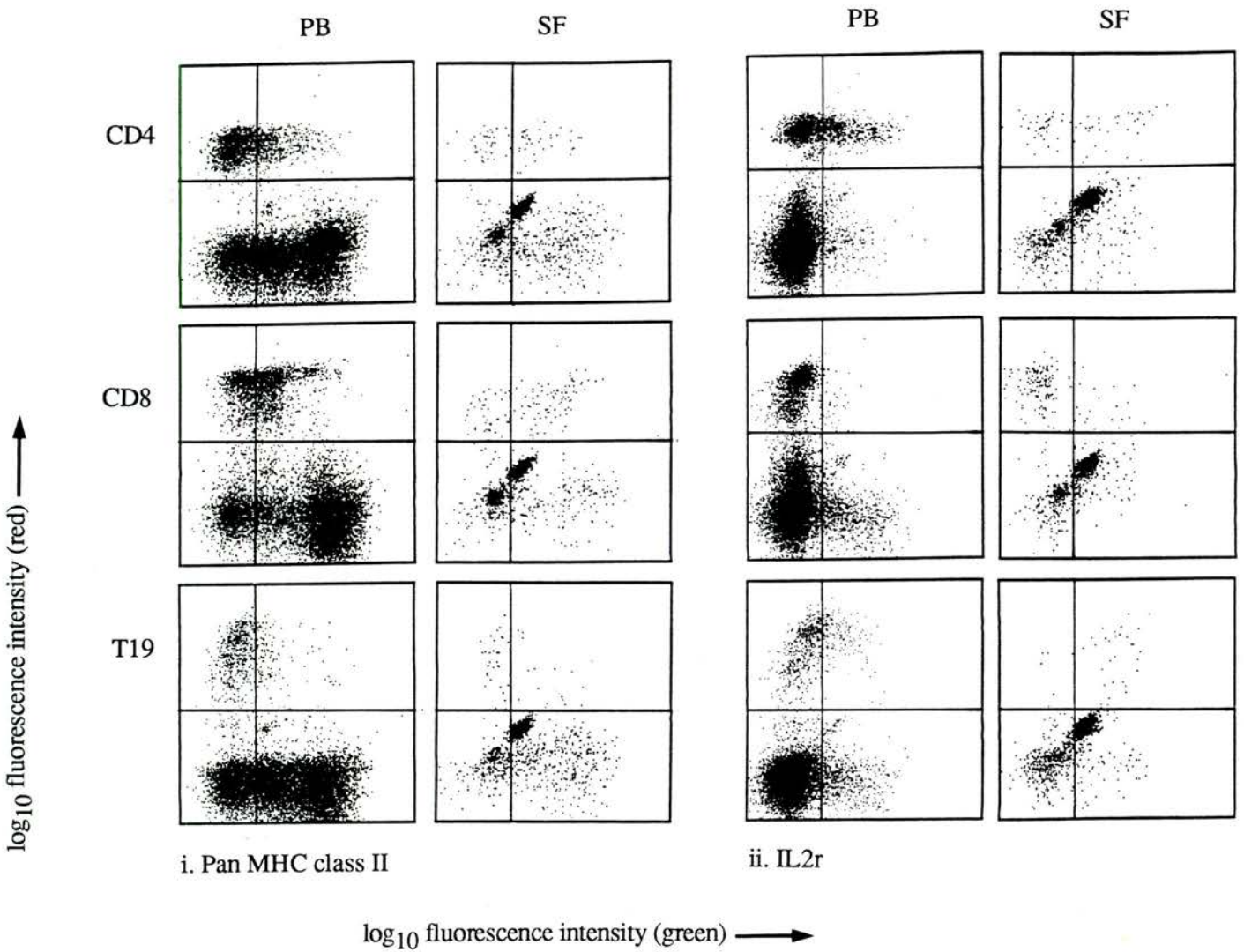

PB
SF
PB
SF
CD4
CD8
T19
log10 fluorescence intensity (red)
i. Pan MHC class II
ii. IL2r
log10 fluorescence intensity (green)

Figure 8.1 and 8.2. Activation status of T lymphocytes in PB and SF from the RC joints of sheep with AIA.

Figure 8.1. MHC class II expression by T lymphocyte subsets in paired PB and SF from OVA- (a) and saline-challenged (b) joints. The percentage values were determined by flow cytometric analysis. The bars indicate the median values and the numbers at the top of each column indicate the number of sheep from which results were obtained. The values obtained from sheep sampled on days 3, 16 and 30 following generation of AIA were pooled for statistical analysis. The statistical significance of results from paired PB and SF are indicated ($^{\dagger}p<0.05$, $*p<0.005$).

Figure 8.2. IL2r expression by T lymphocytes in paired PB and SF from OVA- (a) and saline-challenged (b) joints. The statistical significance of results are as indicated above.

Figure 8.1

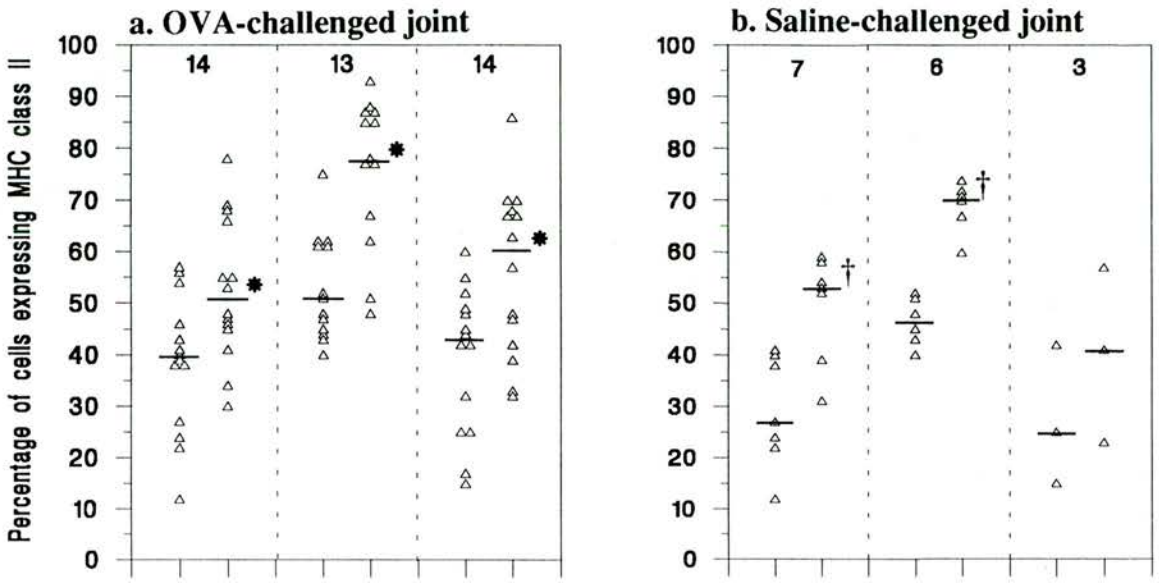
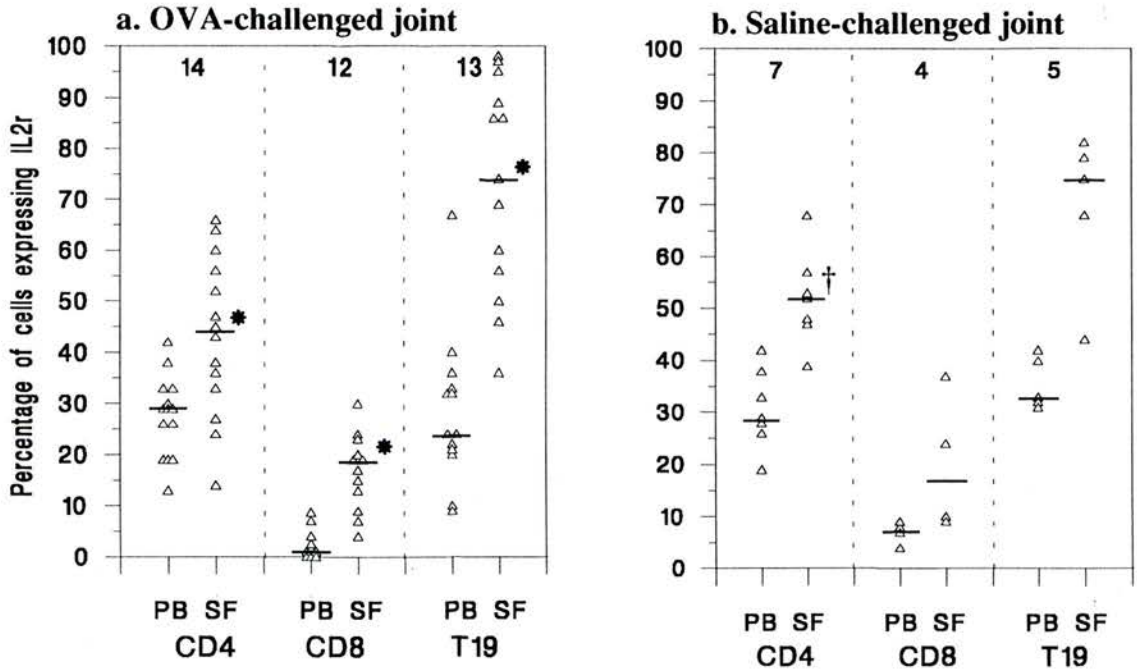


Figure 8.2



was increased in SF compared to PB, paired analysis was not possible because of the insufficient number of samples where analysis was possible. There was no significant difference in the proportions of CD4+ and CD8+ T lymphocytes expressing MHC class II in the right versus the left RC joint. However, the numbers of lymphocytes in SF from the OVA-challenged joint was significantly greater than that from the saline-challenged joint (Section 3.1. and Table 1)

For a smaller number of sheep (n=6), expression of the DQα chain of MHC class II on paired PB and SF T lymphocytes was evaluated (Figure 6). In sheep, expression of this molecule by T lymphocytes is believed to be an indicator of recent activation (Hopkins *et al* 1993). SF was aspirated from the right RC joint of 3 sheep on days 3 and 16 following induction of AIA. These results were pooled for statistical analysis. For each T lymphocyte subset, the proportion of DQα-expressing cells was significantly higher in SF (median values of 47, 74 and 64 for CD4+, CD8+ and γδ T lymphocytes) than paired PB (median values of 26, 20 and 11 for CD4+, CD8+ and γδ T lymphocytes respectively)($p<0.02$ for all 3 subsets).

The above results show that a greater proportion of all T lymphocyte subsets in SF from both joints expressed MHC class II compared to PB and that the level of expression of these activation molecules appeared (although not quantitated) to be higher. The increased numbers of DQα-expressing T lymphocytes in SF from the antigen-challenged joint suggested that these cells had been recently activated.

IL2r expression by T lymphocyte subsets in paired PB and SF was evaluated in the same way as MHC class II expression. These results are shown in Figures 6 and 7. The expression of IL2r was increased on all 3 T lymphocyte subsets in SF compared to paired PB ($p<0.001$). The proportions of each T cell subset expressing this antigen differed significantly from each other ($p<0.002$)(Figure 8). Similar results to those from the right RC joint were obtained for IL2r expression on T lymphocytes from the left RC joint (Figure 7 and 8). Comparison of CD4+ and γδ T lymphocytes from paired PB and SF from the left RC joint showed that the increased expression of this antigen in SF was significant ($p<0.05$). Paired

comparison of IL2r expression on CD4+ and γδ T lymphocytes from right and left RC joints showed no significant differences.

This provided further evidence that a greater proportion of SF T lymphocytes from the right and left RC joints were activated compared to PB T lymphocytes.

3.2.3. Expression of CD45RA and CAM by PB and SF T lymphocytes

To determine the proportion of naive T lymphocytes in SF during AIA, the expression of CD45RA on each T lymphocyte subset in paired SF and PB was determined by double-labelling. Because the proportion of CD45RA-expressing T lymphocytes in PB of adult sheep is relatively small (less than approximately 15%), AIA was generated in 3 lambs aged 8 to 10 months in the same way as described previously. SF was aspirated from the antigen-challenged joint on day 3 and 16 from each animal and the results were pooled for analysis. The results obtained for PB were were similar to previous published values for sheep of this age (Evans *et al* 1994).

These results showed that there were very few CD45RA-expressing T lymphocytes in SF compared to paired PB (Figure 9.1 and 9.2). Although the number of sheep was small there were no obvious differences in the results obtained on day 3 compared to day 16.

In addition, the expression of CD44, L-selectin, β-1 integrin, VLA4 (α-chain) and VLA6 (α-chain) by CD2+ T lymphocytes (CD4+ and CD8+ T lymphocytes) from paired PB and SF was determined in a similar fashion. These results were obtained from 3 sheep (adult) sampled on days 3 and 16 following generation of AIA. Flow cytometry profiles showing the predominant trends in expression of these molecules at both time points are shown in Figure 10.

The expression of these CAM by SF T lymphocytes was variable, with the exception of L-selectin which was always expressed at a lower level (as a percentage of positively-expressing cells and intensity of expression determined from the MFI value) on T lymphocytes from SF compared to PB. On day 3, the

Figure 9.1. CD45RA expression by T lymphocytes in paired PB and SF from sheep with AIA

SF was aspirated from the OVA-challenged joint 3 days following generation of AIA. The expression of CD45RA by CD4+, CD8+ and T19+ T lymphocytes was determined by double-labelling and flow cytometric analysis and compared with these subsets in paired PB. 10,000 cells were counted in each sample.

Figure 9.2. Percentages of CD45RA-expressing cells in PB and SF following generation of AIA.

The percentage values of CD45RA-expressing cells in paired PB and SF from 3 sheep (3 sheep sampled on day 3 and 2 sheep sampled on day 16 following generation of AIA) was determined from flow cytometry. The bars show the median values. The statistical significance of results from paired samples is indicated (*$p<0.03$).

Figure 9.1

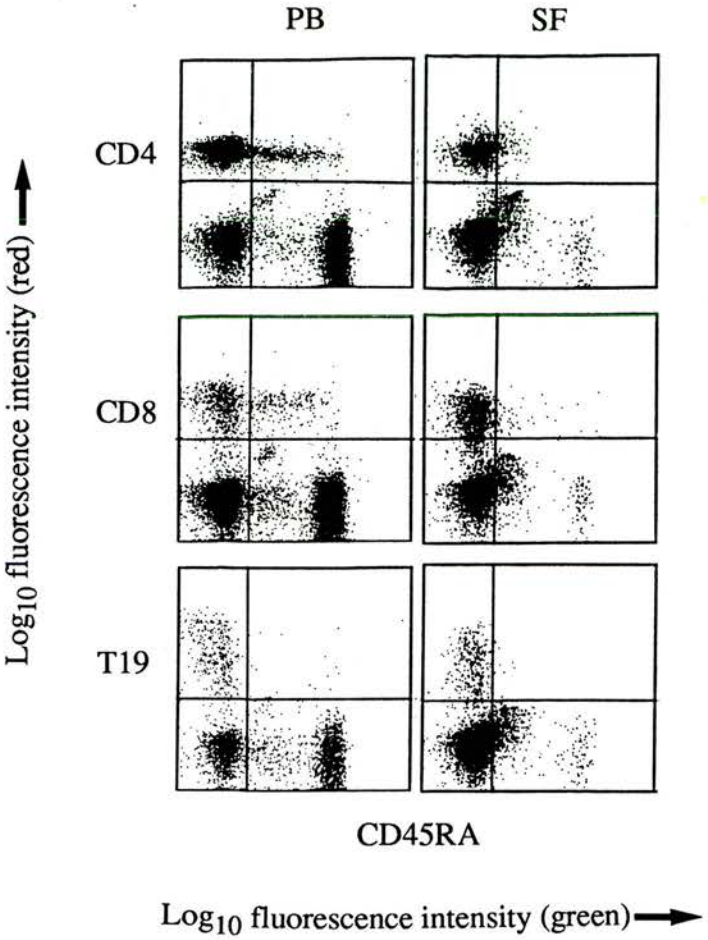


Figure 9.2

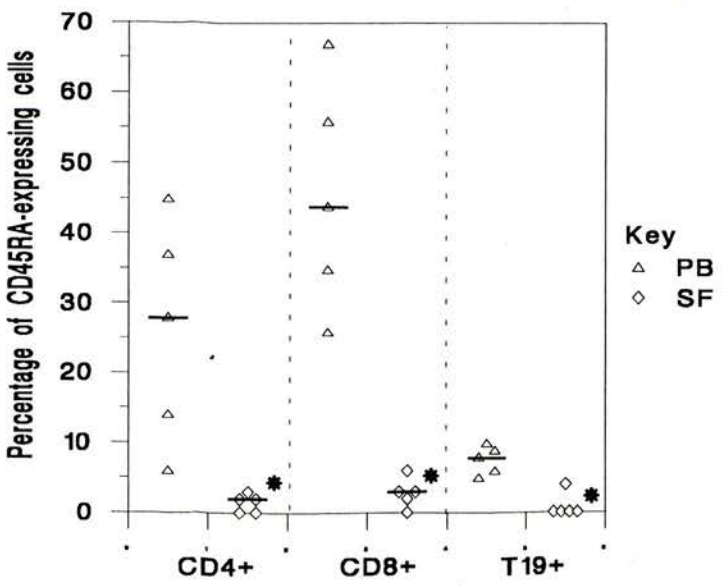
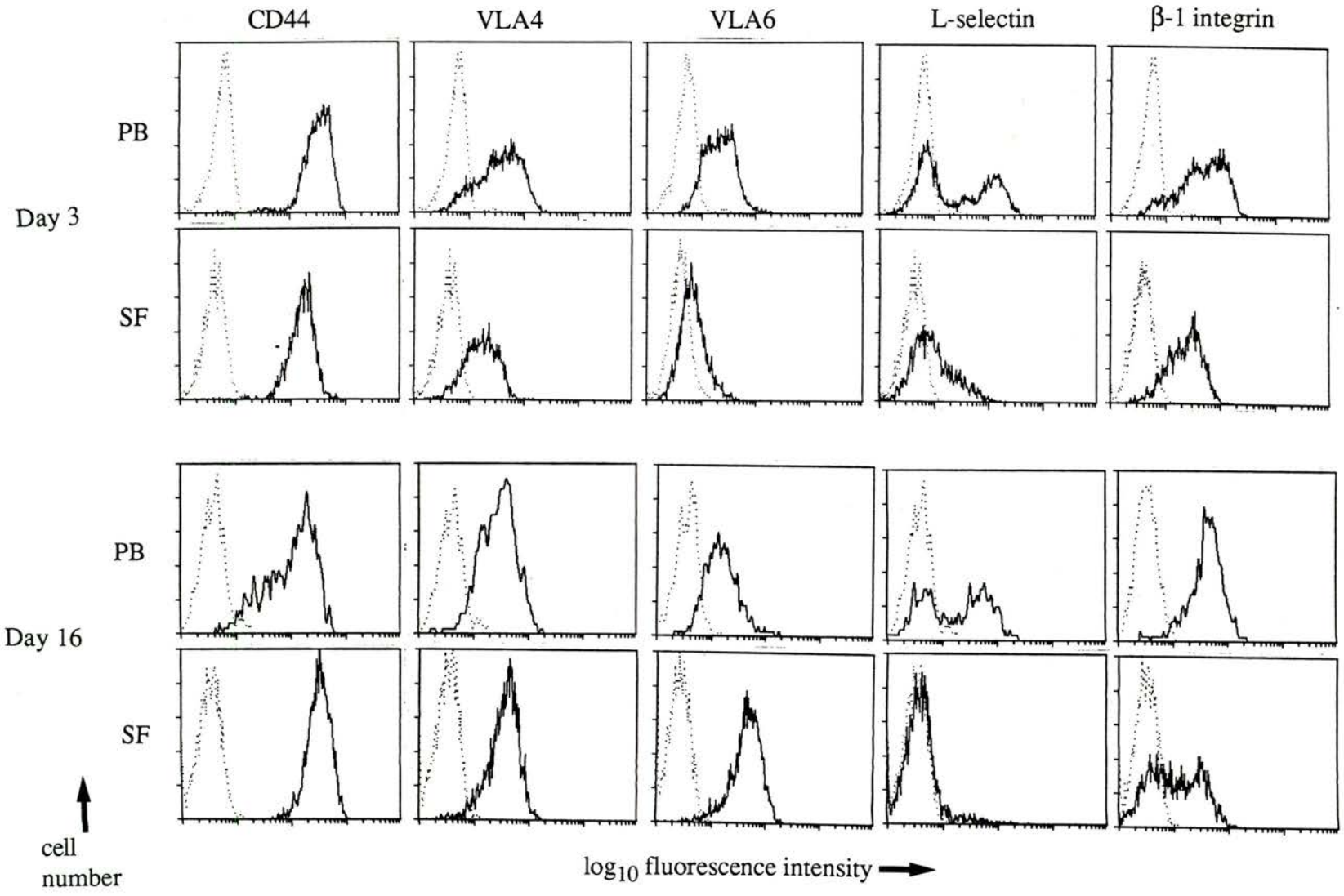


Figure 10. Flow cytometry profiles of CAM expression by CD2+ T lymphocytes in PB and SF following the generation of AIA.

Cells in paired PB and SF on day 3 and day 16 following generation of AIA were incubated with a combination of Mab to CD2 (CD2 is expressed by CD4+ and CD8+ T lymphocytes) and VLA4 (α-chain), VLA6 (α-chain), β-1 integrin, CD44 or L-selectin. The expression of these CAM by CD2+ T lymphocytes was determined by flow cytometry. 5000 cells were counted for each combination of Mab. The dotted line on each graph shows the profile obtained with an isotype-matched control Mab or normal mouse serum (Diluted 1:500) followed by the appropriate FITC-conjugate.

Figure 10


CD44
VLA4
VLA6
L-selectin
β-1 integrin
Day 3
PB
SF
Day 16
PB
SF
cell number
log10 fluorescence intensity

intensity of expression of VLA6 on SF T lymphocytes from all 3 sheep was lower than on T lymphocytes from paired PB. The intensity of expression of the remaining CAM was lower in SF than PB in 2 of 3 sheep but higher in SF than PB in one animal.

On day 16, SF T lymphocytes from 2 of 3 sheep expressed higher levels of VLA6 compared to paired PB. The expression of VLA4 was very similar in both compartments and similar to day 3 whilst β-1 integrin expression was lower on SF T lymphocytes in 2 of 3 sheep. The expression of CD44 by T lymphocytes from SF was higher than from PB in all 3 sheep.

With the exception of L-selectin and β–1 integrin expression, both of which were reduced on T lymphocytes from SF compared to PB at both time points, expression of the other CAM on T lymphocytes in SF was generally lower than on T lymphocytes in PB at day 3, and higher than PB at day 16 following generation of AIA.

Summary of phenotypic characterisation of AIA

1. AIA generated in the RC joint of sheep with 0.5mg OVA resulted in a chronic synovitis of varying severity that persisted for long periods (>12 months) of time in some animals.

2. Phenotypic characterisation of the lymphocytic infiltrate in synovium and SF revealed definite patterns of infiltration that changed during progression of the synovitis. CD4+ T cells predominated over CD8+ T cells on day 3 following generation of AIA and there was a steady decline in the proportions of γδ T cells relative to αβ T cells, and B cells relative to T cells, at the later time points.

3. A large proportion of the infiltrating T lymphocytes were highly activated as judged by MHC class II and IL2r expression.

4. The low proportion of CD45RA-expressing T lymphocytes demonstrated that the vast majority of these cells were memory T cells. Although the number of sheep evaluated was small, the expression of some CAM by T lymphocytes in SF

was variable, with increased and reduced levels of expression of different molecules relative to T lymphocytes in PB at different time points during the progression of AIA.

PART II

T and B lymphocyte responses in AIA

1. T lymphocyte responses during AIA

Previous experience from isolation of PBMC indicated that the number of lymphocytes that could be recovered from SF at any stage during AIA would be too small to perform T lymphocyte proliferation assays using standard protocols. Therefore, to increase the numbers of SF lymphocytes available for these assays flare reactions were induced and larger amounts of antigen were injected into the joint.

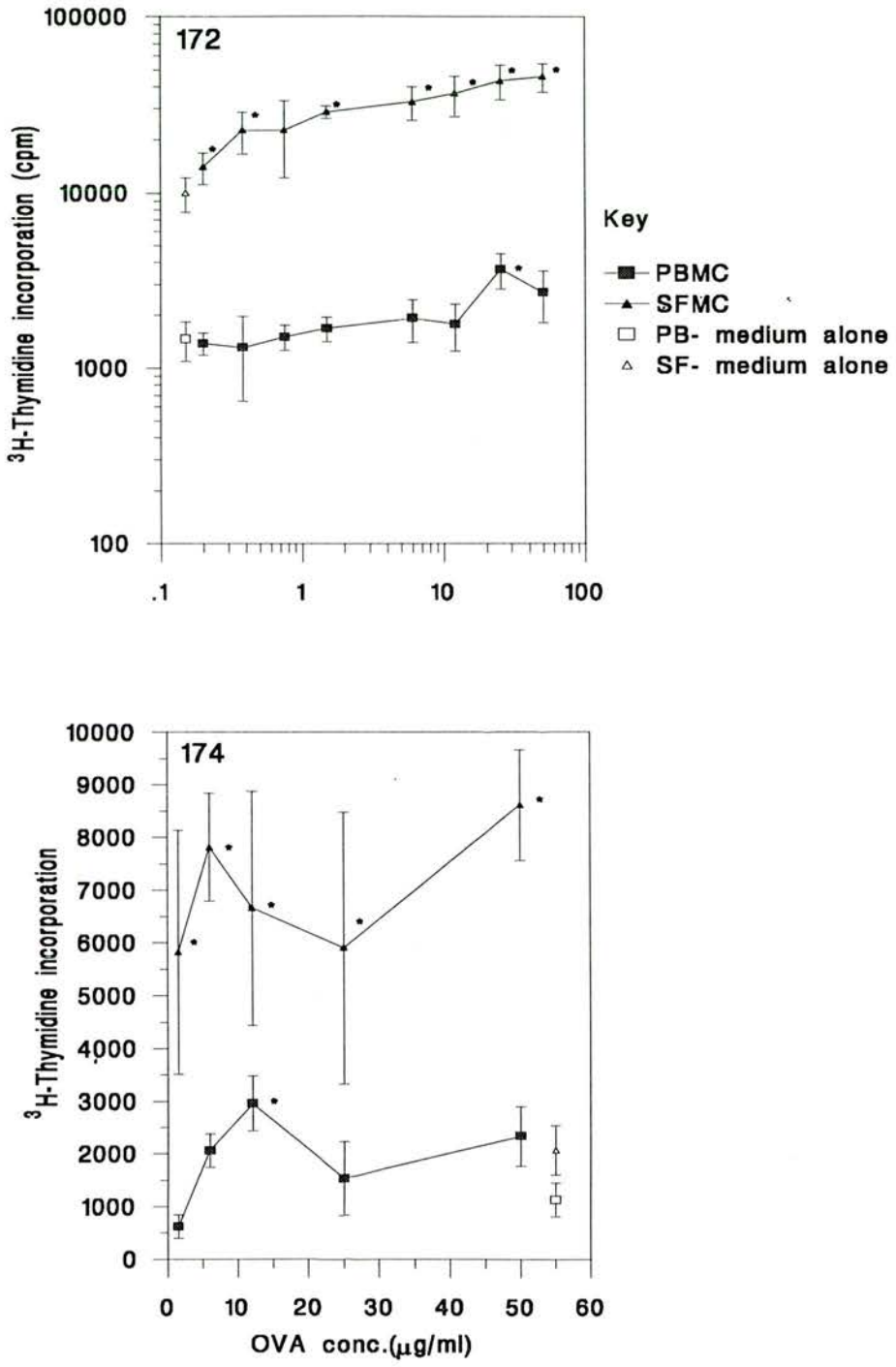
To determine whether OVA-specific T lymphocytes were present in the joint during a flare reaction, 2 sheep (numbers 172 and 174) that 7 months previously had AIA induced in their right RC joints were given a second injection of OVA (2mg) into these joints. PB and SF were collected on days 3 and 16 following induction of the flare response.

Significant proliferative responses were obtained from SF T lymphocytes from both sheep at day 3 with all concentrations of antigen (Figure 1). This was in contrast to the proliferative responses from PB T lymphocytes from both sheep where, in each case, only one concentration of antigen resulted in significant proliferation (Figure 1). At both of these concentrations the proliferative response of SF T lymphocytes was significantly higher than for PB T lymphocytes ($p<0.05$). The high level of proliferation of SF T lymphocytes from sheep 172 with medium alone was suggestive of the presence of antigen on APC acquired *in vivo*. In both sheep the conA responses from SF T lymphocytes were lower than those obtained

Figure 1. PB and SF T lymphocyte proliferation assays from sheep 172 and 174.

Flare reactions were induced by injection of 2mg OVA into the RC joint of sheep 172 and 174. 3 days later paired PB and SF were collected and the mononuclear cells isolated by centrifugation over Lymphoprep. These cells were then incubated in doubling dilutions of OVA in 96-well round-bottomed plates for 5 days. Proliferation was detemined by the incorporation of ^{3}H-Thymidine over the last 5 hours of incubation. The results are expressed as the mean c.p.m. of triplicate cultures ± SD. The * indicates those concentrations of OVA from which significant proliferative responses were obtained compared to medium alone.

Figure 1


172
100000
10000
1000
100
.1
1
10
100
^{3}H-Thymidine incorporation (cpm)
Key
PBMC
SFMC
PB- medium alone
SF- medium alone
174
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0
10
20
30
40
50
60
^{3}H-Thymidine incorporation
OVA conc.(µg/ml)

for PB (Means of 8120 vs 17211 cpm for SF and PB respectively for sheep 174 and, 32798 and 52840 cpm for SF and PB respectively for sheep 172). There were insufficient numbers of cells in SF from both sheep on day 16 to allow proliferation assays to be performed.

The enhanced proliferation of T lymphocytes from SF could have been due an increase in the frequency of OVA-specific T lymphocytes in SF relative to PB or may have resulted from an enhanced efficiency of antigen presentation by APC in SF compared to monocytes in PB.

The paucity of lymphocytes in SF from the left RC joint precluded investigating the possibility that OVA-specific T lymphocytes enter the joint secondary to a non-specific stimulus.

2. B lymphocyte responses

2.1. OVA-specific antibody in RC and TT joints following immunisation

To compare the relative amounts of OVA-specific antibody in serum to SF from the RC and TT joints, Ouchterlony tests were performed with different dilutions of OVA. PB and SF from both RC and TT joints were collected from 4 sheep at the same time point following immunisation to OVA. SF from both RC and both TT joints in each sheep were pooled for analysis. For each sheep the precipitin lines that formed with serum were formed at higher concentrations of OVA (double the concentration in 3 sheep and 4-fold in one)(Figure 2a) than those that formed with SF from either joint. For all sheep the precipitin lines obtained with SF from the RC and TT joints from the same animal formed at the same concentration of OVA (ranging from 8μg/ml to 30μg/ml)(Figure 2a).

This demonstrated the presence of OVA-specific antibody in SF from immunised sheep and showed that there were no major quantitative differences in the concentrations of antibody between RC and TT joints. The volume of SF aspirated from the RC and TT joints of these sheep were very similar (0.5 to 0.8 mls per joint). This showed that the quantity of OVA injected intraarticularly,

Figure 2a. Determination of antibody:antigen equivalence for serum and SF by Ouchterlony tests.

Paired serum and SF from the RC and TT joints were obtained from sheep following immunisation to OVA in CFA. Serum or SF supernatants were pipetted into the central wells and the outer wells were filled with doubling dilutions of OVA solution. Following several days incubation in a humidity chamber the gels were washed in Coomassie blue solution. The dilutions of OVA at which there was antibody:antigen equivalence are shown by arrows. The dilutions of OVA in the outer wells are; 1; 250μg/ml, 2; 125μg/ml, 3; 60μg/ml, 4; 30μg/ml, 5; 15μg/ml and 6; 8μg/ml.

Figure 2b. α-OVA IgG antibody titres in SF from the OVA- and saline-challenged joints following generation of AIA.

18 sheep with AIA had SF aspirated from paired OVA- and saline-challenged joints on day 3, 16 and 30 (6 sheep at each time point) following generation of the disease. Antibody titres were determined by ELISA. The values obtained from paired OVA- and saline-challenged joints are joined.

Figure 2c. α-OVA and α-HSA IgG antibody titres in dual-immunised sheep.

Sheep 175, 179 and 181 were immunised to OVA and HSA in CFA. AIA was generated by injection of 2mg OVA into the right RC joint (RC). Saline was injected into the left RC joint (LC). Serum (S) and SF from both RC joints in each sheep were collected on day 16. Antibody titres were determined by ELISA.

Figure 2a

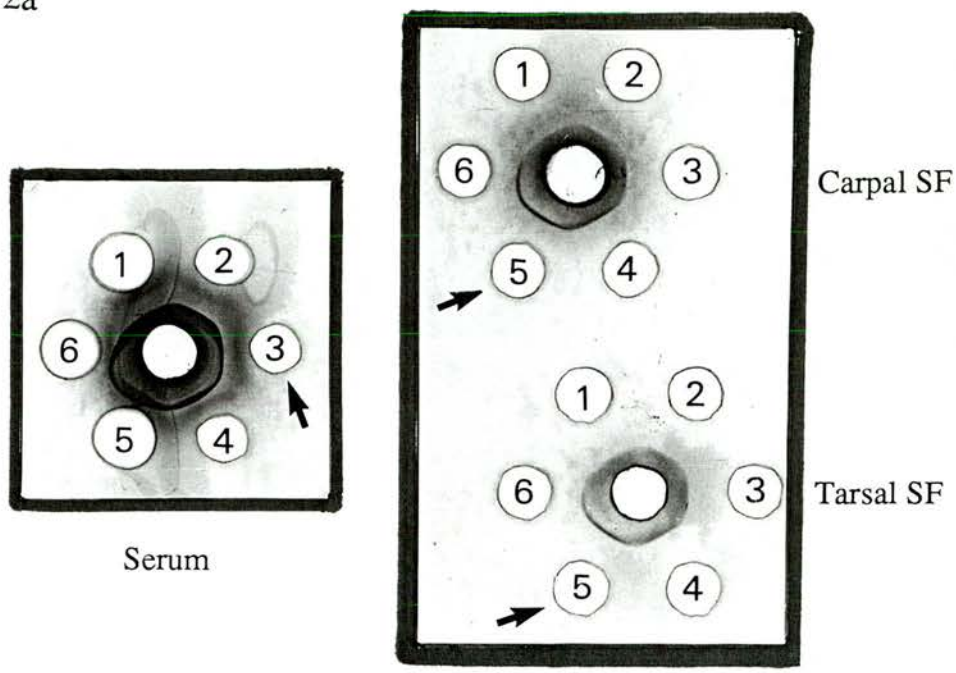


Figure 2b

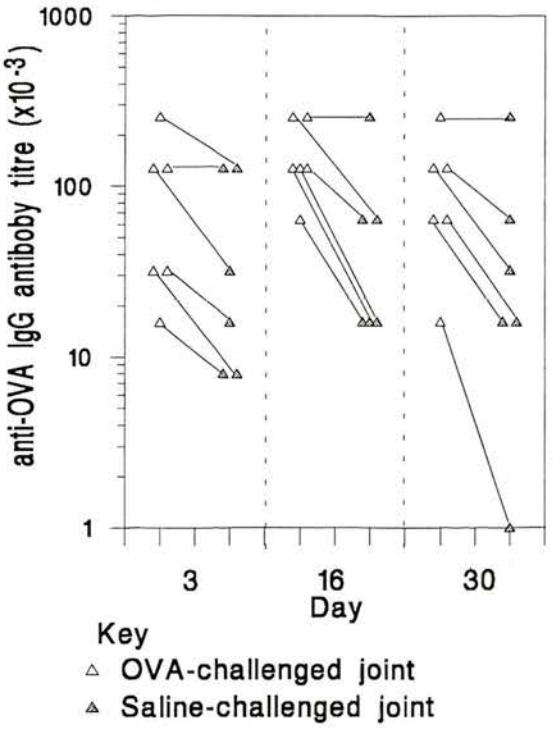
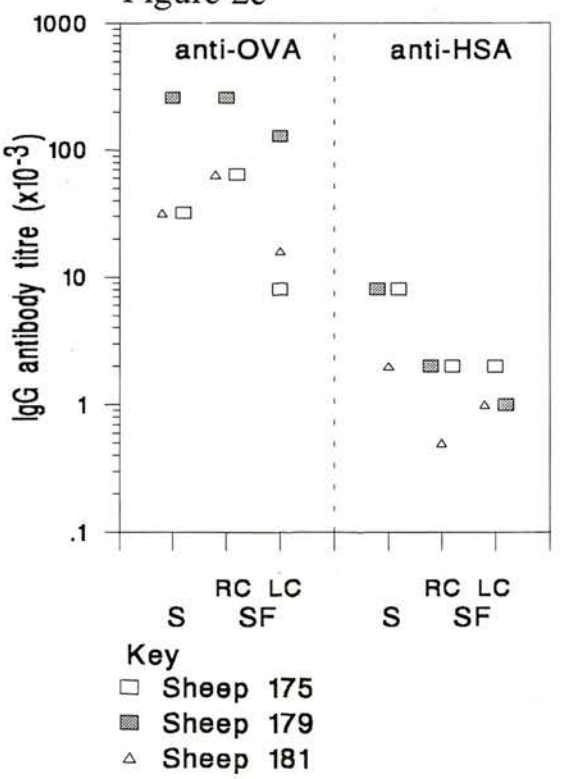


Figure 2c



whether 0.5mg or 2mg, was an excess of antigen relative to the quantity of antibody present in SF.

2.2. Measurement of OVA- and HSA-specific IgG in SF by ELISA during AIA.

Stored supernatants of SF from paired right and left RC joints from sheep at days 3, 16 and 30 following generation of AIA were evaluated by ELISA for IgG antibody to OVA. These results are shown in Figure 2b. The titres of α–OVA IgG antibody in the right RC joint were significantly higher than those in the paired left RC joint at all time points ($p<0.04$). The increased antibody titres in the OVA-challenged joint compared to the paired control joint were, for the majority of sheep, quite small.

Although serum antibody titres were not available for comparison with these SF, the elevated antibody titres in the great majority of OVA-challenged joints compared to paired control joints, together with the variable numbers of plasma cells in the synovium strongly suggested local production of OVA-specific IgG antibody in the joints of some sheep. However, some of the differences in antibody titres between right and left RC joints may have resulted from the effects of chronic synovial inflammation with an associated increase in vascular permeability and leakage of immunoglobulin from the circulation.

To characterise further the specificity of the local B lymphocyte response, 3 sheep (numbers 175, 179 and 181) previously immunised to OVA were immunised to HSA using the same immunisation protocol. AIA was generated in a RC joint using 2mg OVA. PB, SF and synovium were collected from these sheep at post mortem on day 16. The IgG antibody titres to OVA and HSA in serum and SF from both RC joints were measured by ELISA. The results from the 3 sheep are shown in Figure 2c. These show that there was local OVA-specific IgG antibody production in the antigen-challenged joint of sheep 175 and 181. There was no evidence of local HSA-specific antibody production in the OVA-challenged joints

although the antibody titre in the OVA-challenged joint of sheep 179 was elevated in comparison to SF from the saline-challenged joint.

2.3. Detection of OVA-specific B cells and cells containing OVA-antibody complexes in synovium

To demonstrate the presence of OVA-specific B lymphocytes in synovium, cryostat sections of synovium from the right and left RC joints of 6 sheep (3 at day 16 and 3 at day 30) were incubated with serial dilutions of biotinylated OVA (1:500 to 1:10,000 diluted in PBS). All sheep had higher IgG antibody titres to OVA in their antigen-challenged joints compared to their saline-challenged joints. OVA will bind to cells expressing a receptor for OVA ie OVA-specific B lymphocytes, and OVA-specific plasma cells. However, it will also bind to cells containing IC of OVA where these complexes are in antibody excess. This might include macrophages and DC in the synovial lining. Cells that bound OVA were identified by the immunoperoxidase technique using the appropriate reagents. Additionally, synovium from both RC joints of the dual-immunised sheep (n=3) were evaluated in the same way. Control sections were incubated in biotinylated HSA.

Moderate numbers of OVA+ cells were present in synovia from the right RC joint of sheep with AIA generated with 0.5mg OVA (Figure 3). It could not be ascertained with certainty that these positively-stained cells morphologically resembled lymphoid cells. No OVA+ cells were identified in synovia from the left RC joint.

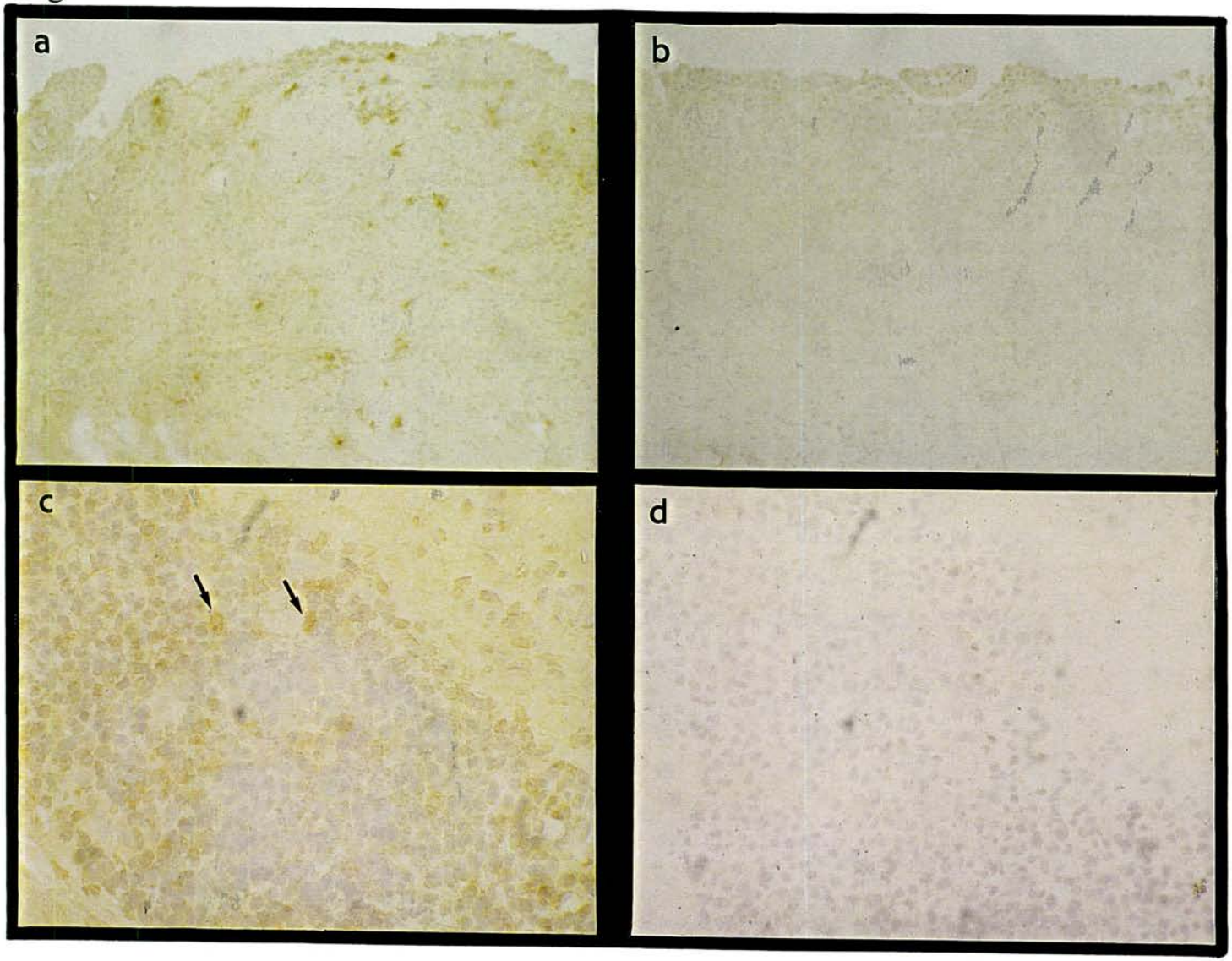
In contrast, large numbers of OVA+ cells were present in synovia from the OVA-challenged joints of dual-immunised sheep. These cells had a lymphoid morphology and were present in large numbers in follicle-like aggregates of cells (Figure 3). The intensity of individual cell staining varied considerably and the majority of cells appeared to have surface membrane staining rather than

Figure 3. OVA-labelled cells in the synovial lining following generation of AIA.

(a). Frozen sections of synovium from sheep with AIA biopsied at day 16 following generation of AIA were incubated in biotinylated OVA (diluted 1:2000). Labelled cells were identified with peroxidase-labelled streptavidin followed by DAB solution. Control sections were incubated in the same dilution of biotinylated HSA (b)(Original magnification x156).

(c). Frozen section of synovium from sheep 181, dual immunised to OVA and HSA. OVA-labelled cells were identified in the same fashion as for the section shown in (a). Control sections were incubated in the same dilution of bHSA (d). Large numbers of positively-labelled cells are present. Two of the more intensely labelled cells are indicated with arrows. (Original magnification x390).

Figure 3



cytoplasmic staining, suggesting that most of these cells were B lymphocytes rather than plasma cells.

Routine histological examination of these tissues revealed the presence of larger numbers of lymphoid cells compared to tissues from sheep with AIA, previously described in Part I (Part I, Figure 5). Large numbers of plasma cells were present in synovia from both sheep that were shown to have elevated SF antibody titres compared to serum (numbers 175 and 181). The majority of lymphoid cells in synovium from the right RC joint of sheep 179 appeared to be small lymphocytes, the number of plasma cells being small by comparison. Very small numbers of HSA+ cells were present in synovium from the OVA-challenged joint of sheep 175. Synovia from the saline-challenged joints of all 3 sheep stained negatively for both antigens (Figure 3).

Summary of T and B lymphocyte responses during AIA

1. OVA-specific T lymphocytes were present in SF during a flare reaction.
2. Elevated levels of OVA-specific IgG antibody were present in SF from OVA-challenged joints compared to control joints up to day 30 following generation of AIA. This together with the presence of plasma cells and OVA+ cells in synovium strongly suggested local synthesis of α-OVA IgG antibody.
3. The results from the dual immunised sheep showed, at one time point, that the B lymphocyte response was specific for the antigen employed to induce the disease. Larger numbers of OVA+ cells were present in synovia when 2mg OVA was used to induce the arthritis compared to 0.5mg OVA.

Discussion

The results obtained in this study confirmed that AIA generated by injecting 0.5mg OVA into a RC joint of sheep resulted in a chronic synovitis that persisted in some sheep for a prolonged period of time. Although there were some

similarities in the histological appearance of synovium comparing the chronic stages of AIA with that from arthritic MVV-infected sheep, there were also some differences. Although not evaluated quantitatively, synovium from the former disease was characterised by larger numbers of plasma cells and appeared to be less richly vascularised compared with most synovia from arthritic MVV-infected sheep. In respect of the former observation, AIA in sheep appears to more closely resemble this disease induced in the rabbit (Dumonde and Glynn 1962) compared to rats or mice, where smaller numbers of plasma cells have been observed (Brackertz *et al* 1977a, Dijkstra *et al* 1987). Additionally, no blood vessels with marked intimal fibromuscular hyperplasia were seen in synovia from any sheep with AIA regardless of the chronicity of the disease.

Recent advances in the understanding of the role of FcR in the initiation of Arthus reactions has shed new light on the pathogenesis of the early stages of AIA. Mice deficient in FcγRI and FcγRIII do not mount an inflammatory response following IgG IC deposition in their skin (Sylvestre and Ravetch 1994). Neutrophil chemotaxis is therefore dependent upon the release of chemoattractants from cells following FcR engagement. However, complement components are necessary to amplify this response in joints because complement depletion attenuates the response (Van Lent *et al* 1992). The cell responsible for the initiation of the inflammatory cascade in skin is thought to be the mast cell (Ravetch 1994). However, no differences were observed in the severity of AIA generated in mast cell deficient mice compared to their normal littermates (Van den Broek *et al* 1987) whereas depletion of phagocytic SLC significantly diminished the inflammatory response (Van Lent *et al* 1993). The results from Chapter 3 showed that SLC express FcγRII and if their expression of the other FcγR parallels that of human synovium (Broker *et al* 1990) it is likely that ovine SLC express all 3 subclasses of FcγR. This suggests that the inflammatory cascade during the early

stages of AIA is initiated by FcγR engagement by IgG IC on SLC and not mast cells.

Immunohistological evaluation of synovium and flow cytometric analysis of the lymphocyte populations in SF showed that there were significant differences in the proportions of the T lymphocyte subsets and B lymphocytes at different time points following the generation of AIA. The reasons for this were not established but may relate to changes in the expression of lymphocyte subset-specific endothelial ligands, changes in subset-specific chemoattractants or preferential retention of subsets at different stages of the disease. Although their numbers were not quantitated, a similar predominance of CD4+ T lymphocytes during the early stages of AIA in the rat has been reported (Verschure *et al* 1989). Although CD8+ T lymphocytes have been identified in synovia of rabbits with AIA (Wilkinson, J. *et al* 1993), immunohistological studies to characterise progression of the synovitis have not been performed in this species. During the chronic stages of AIA and in tissues from the saline-challenged joint CD8+ T cells often predominated over CD4+ T cells. This demonstrated that the predominance of CD8+ T lymphocytes in joint tissues was not a feature unique to MVV-associated synovitis.

The paucity of γδ T cells relative to αβ T cells following the initial acute stage of arthritis suggested that these cells do not play an important role in the chronic stages of AIA. Similar observations were made by Thorpe *et al* (1992) for synovia from sheep with CICIA and their numbers relative to αβ T cells generally were small in MVV-associated synovitis (Chapter 4). Their early appearance at sites of inflammation has previously been shown for experimental Listeria infection in mice (Ohga *et al* 1990) which suggested that these cells form part of an early defense mechanism. It was of interest that the largest numbers of γδ T cells in the joint were recorded at a time when the largest numbers of cells expressing CD1 were observed in synovium. γδ T lymphocytes isolated from the joints of humans with inflammatory arthropathies have been shown to exhibit certain functional

properties *in vitro* including cytotoxicity (Li *et al* 1992) and proliferation in response recombinant human HSP (Hermann *et al* 1992) and HSP derived from Mycobacteria (Holoshitz *et al* 1989) and Enterobacteria (Hermann *et al* 1992). In ruminants, PB-derived γδ T lymphocytes can proliferate *in vitro* to Mycobacterial antigens (Chiodini and Davis 1992, Evans *et al* 1994) and weakly to OVA in secondary immune responses (Evans *et al* 1994). It has also been suggested that these cells may have an immunoregulatory role in preventing the proliferation of antigen-specific CD4+ T cells (Chiodini and Davis 1992). However, the significance of these cells during the chronic stages of AIA is questionable given their paucity relative to αβ T cells.

A greater proportion of all T cell subsets expressed MHC class II and IL2r in SF relative to PB. For MHC class II this increase was particularly pronounced for the CD8+ T lymphocyte subset which parallels observations made of this subset in inflammatory arthropathies in humans (Pitzalis *et al* 1987, Reme *et al* 1990). In spite of their reduced numbers compared to the OVA-challenged joint, the increased proportion of MHC class II-expressing T cells in the saline-challenged joint showed that these changes in activation status may occur irrespective of the stimulus to the joint. Similarly, a higher proportion of all three T lymphocyte subsets expressed IL2r in SF compared to PB. This is in contrast to some observations made of IL2r expression by SF T cells from humans with inflammatory arthropathies such as RA where IL2r expression is not increased (Cush *et al* 1992). These differences could be due to different activation phenomena operating in these disease states or inter-species variation in the activation of these cells. This increase in IL2r expression in SF was particularly pronounced for γδ T cells where, on occasions, almost 100% of these cells expressed IL2r. This suggests that there are either differences in the activation of the different T cell subsets or possibly that the rates of activation differ. The latter is known to be the case for ovine T lymphocytes where γδ T cells express

activation molecules more rapidly following stimulation *in vitro* compared to αβ T cells (Evans *et al* 1994).

The virtual absence of CD45RA expression and the high levels of MHC class II expression by SF T cells suggested that they were memory cells. This is consistent with findings from human arthopathies (Pitzalis *et al* 1988b) and in part explains the exaggerated lymphoproliferation obtained with ST T lymphocytes sheep 172 and 174. In view of this, it was perhaps suprising that there was some variation in the expression of the CAM at the two time points investigated in this study. Memory T cells have been reported to express higher levels of CD44, β-1 integrin and VLA4, and lower levels of L-selectin compared to naive T cells (MacKay 1991). The expression of L-selectin was consistently lower on SF T cells compared PB T cells which corresponds with observations made of these cells from humans with inflammatory arthropathies (Cush *et al* 1992, Takahashi *et al* 1992). Although the number of sheep evaluated was small, the expression of CD44, VLA4, VLA6 and β-1 integrin by SF T lymphocyte was more variable, with a tendency for expression to be lower than that of PB T lymphocytes during the acute stage of AIA (day 3). This is in contrast to reports of expression of these molecules by SF T lymphocytes from inflammatory arthropathies of humans where their expression is elevated compared to PB (Takahashi *et al* 1992). This suggests that during the acute stages of joint inflammation there is cleavage of these receptors from the cell surface. This may either follow engagement of these receptors by their respective ligands or be initiated by factors that stimulate these cells via receptors that are different from the molecule to be cleaved (Brazil 1995). VLA4, VLA6, CD44 and β1 integrin all mediate interactions between lymphocytes and components of extracellular matrix in synovium (Haynes *et al* 1991, Rodriguez *et al* 1992, Nikkari *et al* 1993) which influence proliferative and effector responses of these cells (Miyake *et al* 1993). Additionally, VLA4 has a role in mediating homotypic adhesion (Hemler 1990). Cleavage of these molecules during the acute

stages of inflammation may have the effect of down-regulating T lymphocyte responses and reducing their adhesiveness to tissue components, thereby limiting their accumulation in tissues. This may explain the less organised appearance of the lymphoid cells from the immunohistological studies performed on tissues from day 3 compared to later in the disease when many of these molecules were expressed at higher levels. The levels of expression of the CAM by SF T cells on day 16 were frequently elevated compared to PB which is similar to observations of these cells from inflammatory arthropathies of humans (Takahashi *et al* 1992). However, the expression of β-1 integrin was lower than that found in PB at this later time point. The expression of β-1 integrin by memory T cells in AL draining different tissues in sheep varies (MacKay *et al* 1992b), and the observations made in this Chapter suggest that expression of low levels of β-1 integrin is a feature of memory T cells in joints of sheep. The discordant expression of β-1 integrin and some of the α-chains with which it associates has also been reported for T lymphocytes from humans and may be responsible for distinctive functional or homing capacities of these cells (Horgan *et al* 1992).

The presence of large numbers of MHC class II-expressing cells in the synovia following generation of AIA, many of which appeared to be non-lymphoid, is similar to the findings reported for the rat and rabbit with AIA (Dijkstra *et al* 1987, Verschure *et al* 1989, Wilkinson *et al* 1993b). Although increased numbers of DC in the synovial lining have been reported during the early stages of AIA in the rat (Verschure *et al* 1989), their definitive identification in the absence of DC-specific Mab is somewhat difficult. This problem of identification could have been overcome by the use of combined immunocytochemistry and enzyme cytochemistry (Poulter *et al* 1983). Although CD1-expressing cells were detected in synovia from these sheep, their numbers and the intensity of its expression were not as great as for synovia from some arthritic MVV-infected sheep. Because the function and factors responsible for upregulating CD1 expression in sheep are not

known, the significance of this observation is not clear although it suggests that there may be differences in the activation of cells capable of expressing this molecule between the two disease states.

The SF T lymphocyte proliferation assays confirmed that antigen-specific T cells were present in the joint in the acute phase of a flare reaction. The enhanced proliferative responses of SF T cells compared to PB may be accounted for by the increased proportion of memory T cells in SF and by increased efficiency of antigen presentation by APC in SF compared to PB monocytes (Viner *et al* 1993). The numbers of lymphoid cells in SF following generation of AIA with 0.5mg OVA was generally too low to perform T lymphocyte proliferation assays using standard protocols. However, the numbers of cells would probably have been higher if a larger dose of antigen had been used.

Evaluating whether there is local production of antigen-specific antibody in a joint is usually performed by comparing SF and serum antibody titres. Although the vast majority of α-OVA IgG antibody titres were higher in the OVA-challenged joint at all time points compared to paired saline-challenged joints the differences were frequently small. Moderate numbers of cells stained positively with OVA in synovia from sheep with AIA generated with 0.5mg OVA compared to the 3 sheep that received 2mg OVA. Whether these cells were B cells was not established because double-labelling was not performed. Previous studies to demonstrate antigen localisation in synovium by autoradiography (Van den Berg *et al* 1982) and immunocytochemistry (Hasselbacher *et al* 1980) showed that several days after generating AIA very little antigen was detectable in synovium although Webb *et al* (1972) demonstrated OVA within a small proportion of SF macrophages by immunocytochemistry approximately 4 weeks following generation of AIA. This would suggest that the positively-stained cells in synovia, at least from the later time points, were probably OVA-specific B cells rather than cells containing OVA-antibody complexes. Large numbers of cells in synovium were labelled with OVA

when the higher dose was used to generate the disease. Increasing the dose of antigen used to generate the arthritis is likely to increase the number of antigen-specific lymphocytes in the joint because the inflammation that is generated is more severe. It has been shown previously that six weeks following the generation of AIA antigen-specific antibody production accounts for up to 40% of the local antibody synthesis (Cooke and Jasin 1972). This implies that a large proportion of local antibody production is not specific for the antigen used to induce the arthritis, but its specificity has not been established. Antibodies to proteoglycan and type II collagen have been detected in sera of mice, rats and rabbits following the generation of AIA, albeit at low levels (Champion and Poole 1981, Brauer *et al* 1993a, b), and therefore the possibility exists that some plasma cells in synovia may be producing antibodies to host tissue components.

The results obtained in this Chapter showed that AIA in sheep fulfilled the criteria outlined in the introduction for a model of inflammatory arthritis which would be suitable for studying cell trafficking through an inflamed joint. These investigations are described in the following Chapter.

CHAPTER 6

Antigen-induced arthritis as a model system for studying cell trafficking through an inflamed joint

Introduction

The results from the previous Chapter suggested that AIA in sheep would be an appropriate model system with which to study cell trafficking through an inflamed joint. The characterisation of cells exiting an inflamed joint may provide a unique insight into the dynamics and pathophysiological mechanisms involved in joint inflammation that is impossible to obtain from the characterisation of synovial tissues even if these were obtained at different sequential time points from the same animal during the development of arthritis.

Cells that traffic through tissues, exit via the afferent lymphatic vessels draining those tissues. The cannulation of these lymphatic vessels in humans (Brand *et al* 1993), dogs (Sloop *et al* 1993) and sheep (reviewed in Husband 1990) has allowed the characterisation of cells in AL draining different organ systems and the dynamics of cell trafficking through these tissues to be determined.

Lymphatic vessels have been identified in normal synovium from the joints of several species (Davies 1946, Wilkinson and Edwards 1991). However, recent studies on synovium from humans with RA suggest that lymphatic vessels may be destroyed during chronic synovial inflammation and that synovio-lymphatic fistulae may develop that directly connect the joint space to the lymphatic vessels (Wilkinson and Edwards 1991). If synovio-lymphatic fistulae do exist, this would allow cells in SF to leave the joint without first adhering to components of the synovial lining.

Secondary immune responses in antigen-primed animals results in kinetic and phenotypic changes of T cells trafficking through lymphoid and non-lymphoid tissues (Hopkins *et al* 1986, Kimpton *et al* 1990, MacKay *et al* 1992a, Hopkins *et al* 1993). For non-lymphoid tissues these changes have only been reported for secondary antigenic challenge of skin and similar observations have not been made of lymphocytes draining other tissues during these responses. Additionally phenotypic changes in AL DC draining from sites of secondary antigenic challenge during secondary immune responses have been reported (Hopkins *et al* 1989).

Although DC have been identified in the synovial lining and SF of inflamed joints (Zvaifler *et al* 1985, Iguchi *et al* 1986) their role in the pathogenesis of chronic synovial inflammation is unclear and it is not known whether they perform a similar function to that described for LC in skin ie migration and transportation of antigen to the draining lymph node.

Although the results from the previous Chapter describe the phenotypic characterisation of joint tissues in sheep with AIA generated in the RC joint, cannulation of the prescapular afferent lymphatic ducts is not routinely performed and additionally, these vessels drain a large proportion of the entire forelimb (Saar and Getty 1975). The peripheral afferent lymphatic ducts that are most routinely cannulated in sheep are the prefemoral and popliteal ducts in the hindlimb. Surgical removal of a lymph node results in the anastomosis, several weeks later, of the afferent and efferent lymphatics and the resulting lymphatic vessel is then referred to as a "pseudoafferent" (Hopkins *et al* 1985). The advantage this confers is that the volume of lymph collected is greater and the surgical procedure is easier compared to cannulation of an afferent duct. Because it is not technically possible to cannulate afferent lymphatics that drain a joint with the exclusion of other tissues, the afferent or pseudoafferent lymphatic vessel some distance proximal to the joint must be cannulated. The area drained by the popliteal pseudoafferent lymphatic vessel includes all of the tissues distal to the stifle joint (Saar and Getty 1975), although it would seem likely that most of the cells in the AL will be skin-derived because this is the tissue most likely to encounter antigen and hence is likely to have the greatest throughput of cells. Therefore, if AIA is generated in a TT joint, any cells that traffic through the inflamed joint will be collected together with other cells that have trafficked through the non-inflamed tissues of the distal limb. If these cells differ in character or phenotype and appear in sufficient numbers to be detected over the background level of cell trafficking through the non-inflamed tissues, they should be detected.

There were several aims of the work described in this chapter. The first was to determine whether it was possible to collect AL draining a joint following the generation of AIA and if so to characterise its cellular constituents. Further aims were to monitor the appearance of antigen on cells in AL following its administration and to determine whether cells could migrate from SF into the lymphatics draining the joint.

Outline of methods

Following confirmation that lymph drains from the TT joint to the popliteal lymph node, popliteal lymphadenectomy was performed bilaterally in each of 12 adult sheep, previously immunised to OVA in CFA. The pseudoafferent lymphatic vessel was cannulated and the cellular constitiuents of the AL characterised following overnight collection (approximately 16 hour collection period). The volume of lymph collected was recorded and TNCC and differential cell counts obtained. The lymphoid population was characterised by flow cytometry using Mab to B and T lymphocyte subsets and the expression of activation molecules (MHC class II and IL2r) was determined by double-labelling. The expression of activation and other molecules by AL DC was determined by single-staining flow cytometric analysis. This characterisation of the cellular constituents of AL was performed before and after antigenic challenge of the joint (primary and secondary immune responses and during a flare response). The appearance of antigen on cells in AL was determined following the i/a injection of FITC-labelled antigen and the question of whether cells migrate from SF into the afferent lymphatics draining the joint was addressed by monitoring the appearance of *in vitro*-labelled cells in AL following their injection into the joint space.

Results

1. Lymphatic drainage from the TT joint

To confirm the existence of lymphatic drainage from the TT joint and the route of these lymphatic vessels, 0.5ml of 2% Trypan blue dye was injected into the TT joints of 2 sheep. After 10 minutes these animals were euthanased and the hindlimbs dissected to determine the location of dye-filled lymphatic vessels. Figure 1 shows that afferent lymphatic ducts from the joint, containing dye, drain to the popliteal lymph node and to a lesser extent up the medial aspect of the limb into the inguinal region. This confirmed that the popliteal pseudoafferent lymphatic duct would be suitable to characterise the AL draining from the TT joint.

2. Cannulation of the popliteal pseudoafferent lymphatic duct

17 attempts to cannulate the popliteal pseudoafferent lymphatic vessel were made in 12 sheep. The outcome of these procedures is shown in Table 1. To characterise the changes in AL during a flare reaction, 3 sheep had AIA generated 4 to 8 weeks prior to cannulation (see section 7). Of the remaining 14 cannulation attempts, only 3 (21%) flowed for a sufficient period of time to allow data to be collected prior to, and following antigenic challenge of the joint. Lymph was allowed to flow for a minimum of 3 days before the results were included in the analysis. This allowed any inflammation associated with the surgical procedure to settle. AL was collected from sheep 139, 172, 173 and 180 for 7 weeks, 2, 4 and 8 days respectively following antigenic challenge. The AL was collected until the cannula became blocked with fibrin clots or until it was pulled out by the sheep.

3. Characterisation of AL prior to antigenic challenge of the joint

The cellular composition of AL from sheep 139, 172 and 173 prior to antigenic challenge is shown in Table 2. The 2 main cell types identified on cytospin preparations were lymphocytes and DC. Flow cytometric analysis confirmed that most of these lymphocytes were CD4+ T cells, with smaller

Figure 1. Lymphatic drainage from the TT joint

0.5ml of 2% Trypan blue was injected into the TT joint of a sheep sedated with xylazine. After 10 minutes the sheep was euthanased and the hindlimb dissected to determine the course of the lymphatic drainage from this joint. On the medial aspect of the limb (a) one small lymphatic duct can been seen arising from the TT joint (open arrow) and entering the inguinal region proximally. On the lateral aspect of the limb (b), lymphatics filled with blue dye (arrowed) can be seen from the the TT joint to the popliteal lymph node (curved arrow).

Figure 1

Table 1. Outcome of surgeries to cannulate the popliteal pseudoafferent lymphatic duct

Outcome of surgery	Number
Successful cannulation of lymphatic duct with flow of lymph maintained following generation of AIA or flare reaction	4 (24%)
No / inadequate lymphatic ducts	6 (36%)
Lymphatic duct cannulated but lymph flow stopped prior to generation of AIA, or cannula pulled out by sheep	7 (40%)
Total	17

Table 2. Cellular composition of AL

Cell type[a]	Mean % (n=3)	Range (n=3)
Neutrophil	1	(1)
DC	9	(8.5-9.5)
Lymphocyte	90	(89-90.5)
CD4	61	(54-68)
CD8	14	(13-15)
T19	9	(7-11.5)
B lymphocyte	5	(2-8)

[a] The % values for neutrophils, DC and lymphocytes were derived from Leishman's-stained cytospin preparations. The percentage values for the B and T lymphocyte subsets were obtained by flow cytometric analysis of the lymphocyte population.

numbers of CD8+ T, γδ T and B cells. Several days after surgery neutrophils were present in very small numbers and occasional eosinophils and cells undergoing division were seen on cytospins of AL from all sheep. Large basophilic lymphoblasts were occasionally observed but they never comprised more than 2% of the total lymphocyte population. The basic composition and proportions of the lymphocyte subsets were similar to published values for popliteal pseudoafferent lymph (MacKay *et al* 1988a, Kimpton *et al* 1990).

4. Characterisation of AL following antigenic challenge

To characterise the changes in the composition of AL following antigenic challenge of the joint, the TT joints of the hindlimb previously cannulated in sheep 172 and 139 were injected with 0.5mg OVA. To determine the changes following primary antigenic challenge, the TT joint of the hindlimb previously cannulated in sheep 173 was injected with 0.5mg HSA.

To monitor the appearance of antigen on cells in AL, both antigens were FITC-labelled prior to injection (section 4.6). AL was collected at 30 minutes, 60 minutes, 2, 4, 8, 12 and 24 hours following antigenic challenge in sheep 139. Because of the smaller volumes of lymph collected from sheep 172 and 173, the first collection time for these sheep were 8 and 2 hours respectively. Thereafter, AL was analysed following overnight collection.

4.1. Volume of AL

The volume of lymph collected on an hourly basis could only be accurately determined for sheep 139 (secondary antigenic challenge) because of the frequent problems associated with blockage of the cannulae with fibrin clots in sheep 172 and 173. Following antigenic challenge, the volume of AL (ml/hr) collected from sheep 139 increased from a pre-challenge level of approximately 5 mls/hr to a peak of 10mls/hr at 4 hours post-challenge. Although there was minor fluctuation in the output thereafter, it remained above pre-challenge levels until day 8 post-challenge.

4.2. Cellular composition

Cell concentrations in AL were calculated from the TNCC and the differential cell count obtained from Leishman-stained cytospin preparations. Because the output of AL could be accurately determined for sheep 139 (secondary response), the total cell outputs were also calculated based on these figures and the hourly volume of AL collected. The changes in composition of AL following primary (sheep 173) and secondary (sheep 139) antigenic challenge are shown in Figures 2 and 3.

4.2.1. Primary antigenic challenge (sheep 173)

The most notable early change following primary antigenic challenge was an increase in the numbers of neutrophils (Figure 2a). Their concentration peaked at 8 hours following antigenic challenge although as a percentage of the cells in AL, they reached their peak at 12 hours (28%). Their numbers returned to pre-challenge levels on day 3. The concentration of DC was increased at 24 hours post-antigenic challenge and this returned back to pre-challenge levels by day 3 (Figure 2a). There was also an increase in the total lymphocyte concentration on day 1 that returned to pre-challenge levels by day 3 (Figure 2b). However, the concentration of these cells was increasing prior to antigenic challenge and the significance of the increase following challenge is questionable. The proportion of large basophilic lymphoblasts did not change compared to pre-challenge levels (remained at 2% or less of the total lymphocyte population). The percentages of the B and T lymphocyte subsets throughout the course of the experiment remained constant (data not shown).

4.2.2. Secondary antigenic challenge (sheep 139 and 172)

Following secondary antigenic challenge in sheep 139, the concentration of neutrophils was increased above pre-challenge levels 1 hour after antigenic

Figure 2. Cellular composition of AL from the popliteal pseudoafferent lymphatic following primary antigenic challenge (HSA) of the TT joint (sheep 173).

Figure 2a. DC and neutrophils.

Following overnight collection the concentrations of DC and neutrophils in AL were determined from the differential cell count, obtained from a Leishman's-stained cytospin, and the TNCC.

Figure 2b. Lymphocyte populations.

The total lymphocyte concentration was determined in the same fashion as indicated above for neutrophils and DC. The concentrations of the B and T lymphocyte subsets were calculated from these values and the percentage values obtained from flow cytometric analysis of this population analysed on each day. The position of the live gate used to analyse this population is shown in Chapter 2, Figure 2.1c. The arrows indicate the time of antigenic challenge of the joint (0.5mg HSA).

Figure 2

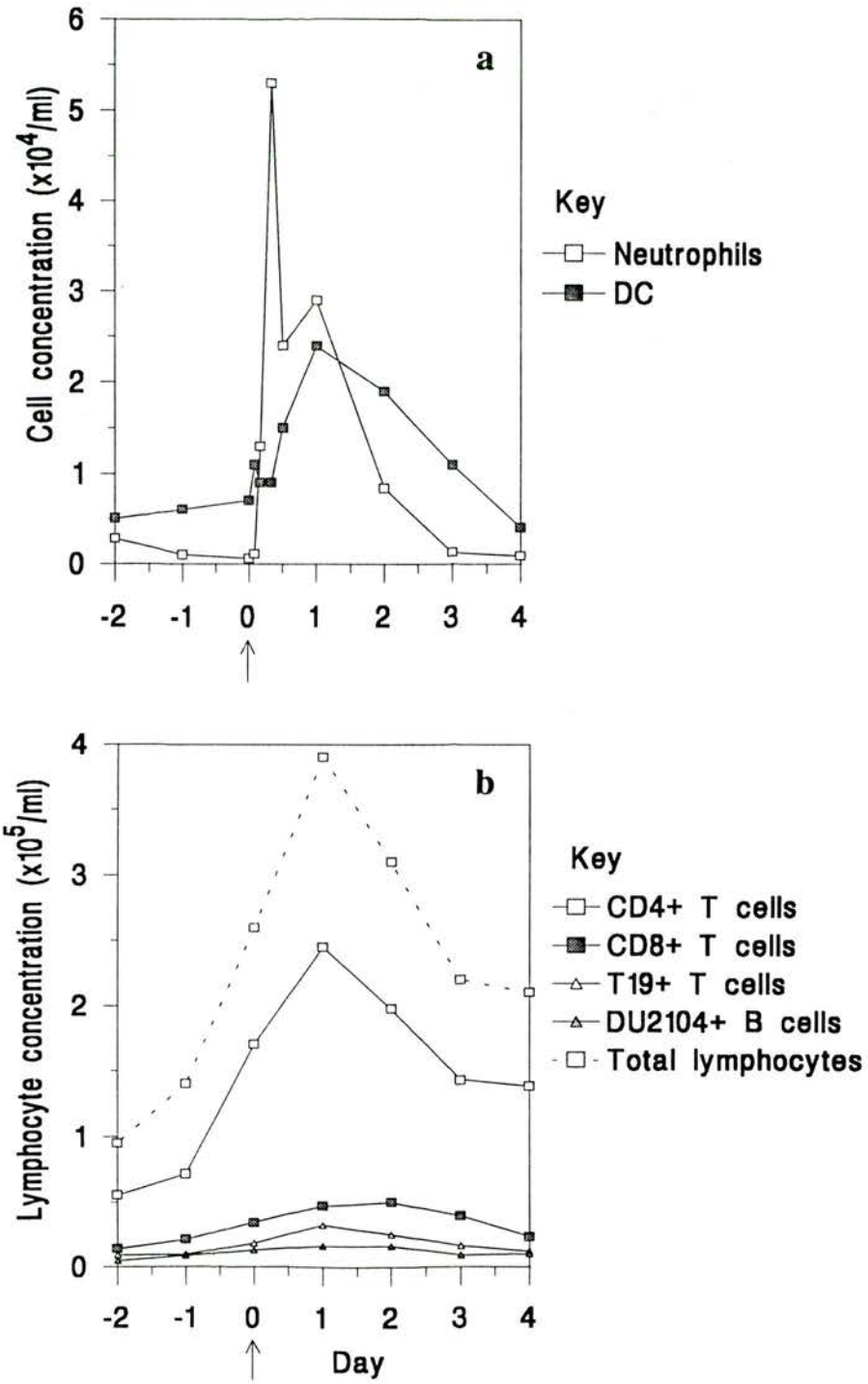
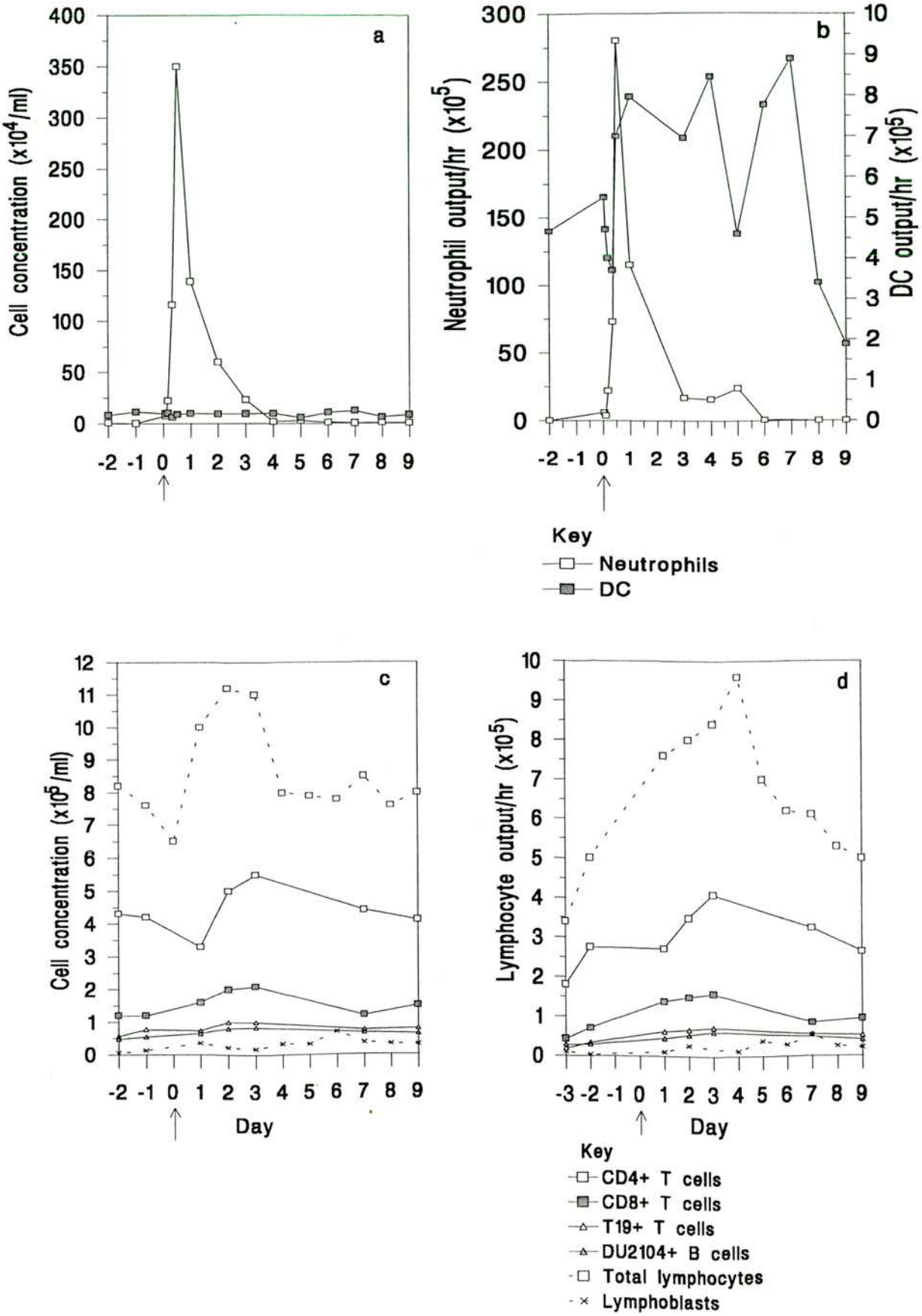

a
Cell concentration (x10⁴/ml)
6
5
4
3
2
1
0
-2
-1
0
1
2
3
4
Key
Neutrophils
DC
b
Lymphocyte concentration (x10⁵/ml)
4
3
2
1
0
-2
-1
0
1
2
3
4
Day
Key
CD4+ T cells
CD8+ T cells
T19+ T cells
DU2104+ B cells
Total lymphocytes

Figure 3. Cellular composition of AL from the popliteal pseudoaffferent lymphatic vessel following generation of AIA in the TT joint (sheep 139).

Figure 3a and b. The concentations of neutrophils and DC were calculated from the differential cell counts (Leishman-stained cytospin) and the TNCC of AL following overnight collection. The cell outputs (cells/hour) were calculated from these values and the hourly volume of AL collected over this period. Arrows indicate the time of antigenic challenge of the TT joint (0.5mg OVA).

Figure 3c and d. The concentrations of the B and T lymphocytes in AL were calculated from the differential cell counts, TNCC and the percentage values obtained following daily flow cytometric analysis of these cells. The cell outputs were determined in the same fashion as indicated above for neutrophils and DC. The position of the live gate used to analyse the lymphocyte population is shown in Chapter 2, Figure 3.1c.

Figure 3


a
Cell concentration (x10⁴/ml)
400
350
300
250
200
150
100
50
0
-2 -1 0 1 2 3 4 5 6 7 8 9
b
Neutrophil output/hr (x10⁵)
DC output/hr (x10⁵)
300
250
200
150
100
50
0
10
9
8
7
6
5
4
3
2
1
0
-2 -1 0 1 2 3 4 5 6 7 8 9
Key
Neutrophils
DC
c
Cell concentration (x10⁵/ml)
12
11
10
9
8
7
6
5
4
3
2
1
0
-2 -1 0 1 2 3 4 5 6 7 8 9
Day
d
Lymphocyte output/hr (x10⁵)
10
9
8
7
6
5
4
3
2
1
0
-3 -2 -1 0 1 2 3 4 5 6 7 8 9
Day
Key
CD4+ T cells
CD8+ T cells
T19+ T cells
DU2104+ B cells
Total lymphocytes
Lymphoblasts

challenge (5% of the cells in AL at this time)(Figure 3a). Their concentration and output peaked at 12 hours (80% of the cells in AL) after which time their numbers gradually fell, returning to normal on day 4 (Figure 3a and b). For sheep 172 (data not shown), an insufficient volume of lymph collected during the first 4 hours post-antigenic challenge precluded analysis of its composition and the frequent problems associated with clots in the cannula made interpretation of the results more difficult. However, at 8 hours following antigenic challenge neutrophils comprised 34% of the cells in AL. Their numbers fell more rapidly compared to sheep 139 and were at pre-challenge concentrations on day 2. The concentrations of DC in the AL of sheep 139 (Figure 3b) and 172 remained relatively constant although their output in the AL of sheep 139 was increased following antigenic challenge and this remained elevated for about 7 days (Figure 3b).

The percentages of the B and T lymphocyte subsets remained constant throughout the experiments in both sheep, with the exception of CD4+ T lymphocytes in the AL of sheep 139 at day 1 post-antigenic challenge (data not shown). At this time the percentage value for these cells fell from a pre-challenge level ranging from 54 to 55% down to 36%. This resulted in the total of the percentages for the lymphocyte subsets at this time point equalling 67% of the cells in the FACS lymphocyte "live" gate. The reasons for this were unclear. Flow cytometric analysis was not performed on the day of antigenic challenge in sheep 139 because the sheep was located some distance from the Department of Veterinary Pathology, Edinburgh, and because of the requirement to analyse the AL at time points throughout the day. The concentration and total output of all lymphocyte subsets in the AL increased between day 1 and day 3 (Figure 3c and d). Although flow cytometric analysis was not performed between days 4 and 6 in this sheep, the total lymphocyte concentration had returned to pre-challenge levels by day 4. However, because of the increased volume of lymph collected on day 4 the peak output of lymphocytes was recorded at this time (Figure 3d). For sheep 139 the highest concentration and output of large basophilic lymphoblasts was

recorded on day 6 (8% of lymphocytes)(Figure 3d). Calculation of CD4:CD8, αβ:γδ and T:B ratios at each time point showed minimal fluctuation throughout the course of the experiment (data not shown).

In the AL from sheep 172 there was an increase in the concentration of lymphocytes on day 2 following antigenic challenge compared to pre-challenge levels (approximately 40% increase over pre-challenge concentrations))(data not shown). However, the proportion of large basophilic lymphoblasts was not increased compared to pre-challenge levels (2% of the lymphocytes in AL).

Although it is difficult to draw conclusions from such a limited number of sheep, the results from sheep 173 (primary) and 139 (secondary) do suggest that there are some similarities and differences in the changes in AL following primary or secondary immune challenge of a joint. In both cases, antigenic challenge of the joint resulted in the early appearance of large numbers of neutrophils and an increase in the concentration or output of DC. In both sheep there were increased concentrations of lymphocytes in AL following antigenic challenge that peaked at levels approximately 50% above those present at the time of challenge. However, these peak concentrations were recorded on day 1 following primary immune challenge and on day 3 following secondary immune challenge of the joint.

The question arises as to whether the increased concentrations/outputs of these cells were due to trafficking of increased numbers of cells through the joint or through adjacent tissues. Whilst every effort was made to inject antigen into the joint space, it is possible that leakage through the tract left by the needle could have resulted in a mild DTH response in the adjacent skin, although this was not clinically apparant in any of the sheep. However, the magnitude of the acute inflammatory response in sheep 139 would suggest that the changes observed were due to inflammatory events in the joint.

4.3. Activation status of T lymphocytes in AL

4.3.1. Primary antigenic challenge

Following primary antigenic challenge, there were minor fluctuations in the percentages of T cells expressing MHC class II and IL2r (Figure 4.1a and 4.2a). Overall there was little change in the level of expression of these molecules compared to pre-challenge levels (Illustrated for CD4+ T lymphocytes in Figure 4.1a).

4.3.2. Secondary antigenic challenge

For sheep 139, there was a reduction in the number of cells expressing MHC class II and IL2r (Figure 4.1b and 4.2b) and in the level of expression (shown for CD4+ T cells in Figure 4.1b) by T lymphocyte subsets in AL from day 1 to day 3 post-antigenic challenge. However, the percentages of MHC class II+ T cells and their level of expression of this molecule on day 7 were higher than pre-challenge levels (Figure 4.2b). However, the concentration of lymphocytes in AL on day 7 was at pre-challenge levels (Figure 3b) suggesting that there were few or no joint-derived lymphocytes collected at this time. IL2r expression was not elevated on day 7 (Figure 4.1b). The changes in expression of MHC class II and IL2r by lymphocytes on days 1 and 2 post-challenge in sheep 172 were very similar to those observed at these time points in sheep 139 (data not shown).

4.4. Expression of CAM and CD45RA by lymphocytes in AL

The expression of CD45RA, L-selectin, β-1 integrin, VLA4, VLA6, and CD44 was assessed by single-staining flow cytometric analysis of lymphocytes in AL following primary (173) and secondary (172) antigenic challenge (CD45RA expression was also determined for lymphocytes in AL of sheep 139). Although this analysis included B and T lymphocytes, the percentages of the former were 1 to 2% in the AL of sheep 172 and 4 to 7% in the AL of sheep 173 showing that

Figure 4.1 and 4.2. Activation status of T lymphocytes in AL from the popliteal pseudoafferent lymphatic vessel following primary (sheep 173) or secondary antigenic challenge (sheep 139) of the TT joint.

Figure 4.1. Flow cytometry profiles of CD4+ T lymphocytes from AL of sheep 173 (a) and sheep 139 (b) labelled with Mab to MHC class II (pan MHC class II reagent) or IL2r before, and at various time points following antigenic challenge of the TT joint. The dotted line shows the level of autofluorescence obtained with either an isotype-matched control antibody or normal rat serum (diluted 1:500)

Figure 4.2. Percentages of the T lymphocyte subsets positively-labelled with Mab to MHC class II and IL2r in AL from sheep 173 (a) and sheep 139 (b). These values were obtained from double-labelling flow cytometric analysis. The arrows indicate the time of antigenic challenge of the joint.

Figure 4.1

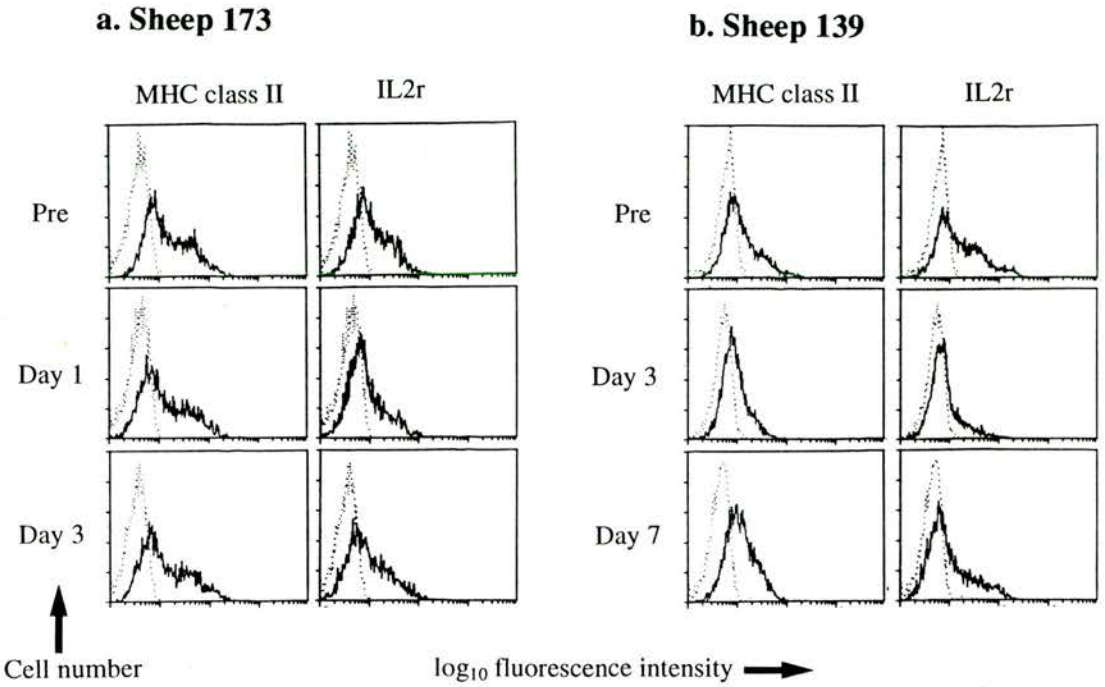
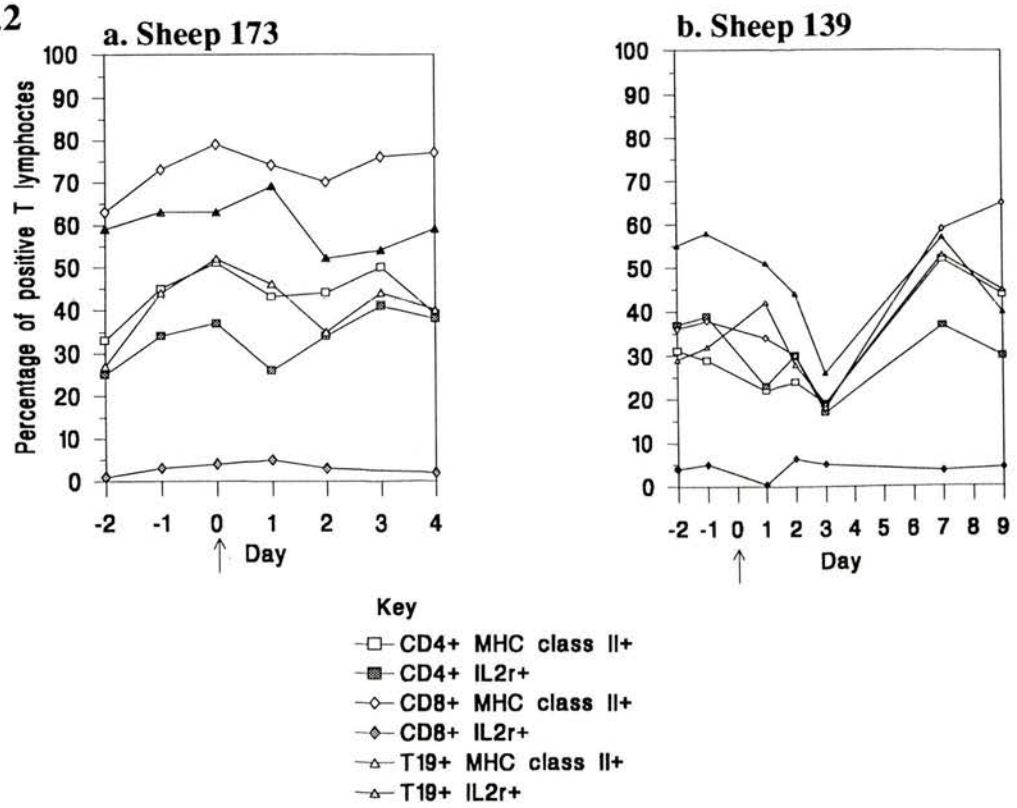


Figure 4.2



the results essentially reflected the expression of these molecules by T lymphocytes. These results are shown in Figure 5.

4.4.1. Primary antigenic challenge

There was very little change in the percentages of cells expressing the above molecules over the 4 days following primary antigenic challenge (Figure 5a). However, changes in the level of expression of these molecules were observed. For L-selectin, CD44, VLA4, and β-1 integrin there was an increase in the level of expression on day 1 post-antigenic challenge which fell back to pre-challenge levels by day 3. On day 1 post-antigenic challenge the percentage of L-selectin^{hi} cells ($>10^2$ fluorescence intensity) increased from pre-challenge levels of 14% to 31%. The proportion of L-selectin^{hi} cells subsequently returned to pre-challenge levels by day 4. This shifting pattern of expression coincided with the increased concentration of lymphocytes in AL on day 1 and 2.

4.4.2. Secondary antigenic challenge

For sheep 172 there were no changes in the percentages or level of expression of any of these molecules on day 2 post-antigenic challenge compared to pre-challenge levels, with the exception of L-selectin (Figure 5b). The proportion of lymphocytes expressing this molecule increased from pre-challenge levels of 60% to 70% on day 2 post-challenge. This was associated with an increase in the number of lymphocytes expressing high levels of this molecule ($>10^2$ fluorescence intensity)(increased from 21% immediately prior to antigenic challenge to 34% on day 2). For sheep 139 the percentage of CD45RA+ cells remained constant throughout the experiment (data not shown).

If the increases in the concentrations of lymphocytes in AL following antigenic challenge were due to joint-derived lymphocytes, their expression of activation and other molecules may differ from those cells that have trafficked through normal

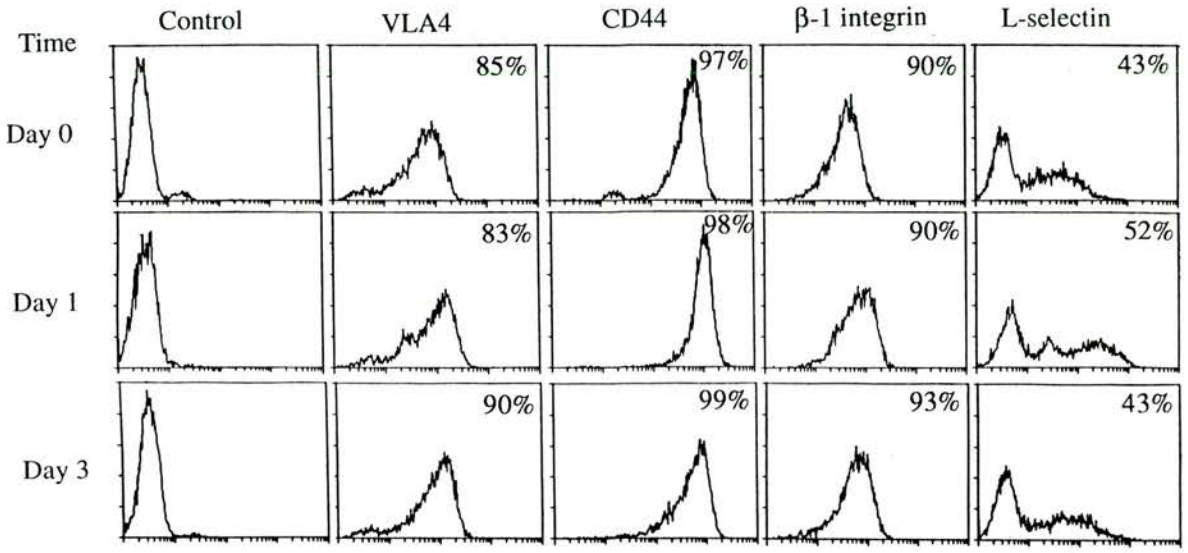
Figure 5. Expression of CAM by lymphocytes in AL from the popliteal pseudoafferent lymphatic vessel at various time points following primary (a: sheep 173) and secondary antigenic challenge (b: sheep 172) of the TT joint.

Figure 5a. Flow cytometry profiles of lymphocytes in AL labelled with Mab to VLA4 (α-chain), CD44, β-1 integrin and L-selectin. The percentage values shown indicate the proportion of cells positively-labelled with each Mab.

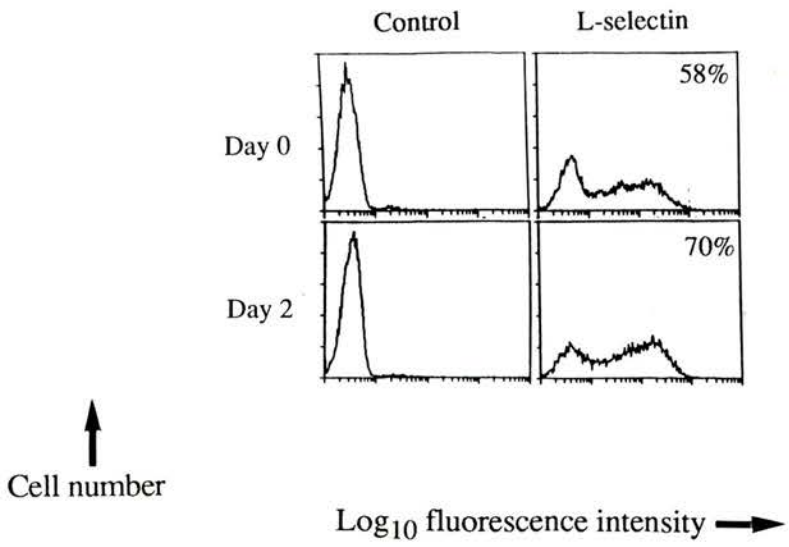
Figure 5b. Flow cytometry profiles of lymphocytes in AL labelled with Mab to L-selectin. The percentage values shown indicate the proportion of cells positively-labelled with each Mab.

Figure 5

a. Sheep 173



b. Sheep 172



tissues. Conversely, the expression of these molecules would not be expected to change on cells trafficking through the non-inflamed tissues. Increased concentrations of lymphocytes were present in the AL at various time points following primary and secondary antigenic challenge. However, for both situations, the maximum increase in concentrations suggested that up to approximately 35% of the cells collected could have been joint-derived. Generally, with the exception of L-selectin expression, the changes in the expression of activation and CAM affected the entire population of lymphocytes collected. This would suggest that the changes observed may have resulted from factors affecting all collected cells These could include:

1. Soluble factors released from the inflamed joint that entered the lymph.
2. Cell-cell interactions in the collecting bottle.
3. Factors released from cells that had trafficked through the joint influencing the phenotype of non-joint-derived cells.

4.5. Phenotypic changes in AL DC following antigenic challenge

The expression of MHC class II (DRα and DQα), CD1 and CD44 on AL DC following secondary antigenic challenge (sheep 139) was determined by flow cytometric analysis. Analysis of AL DC from sheep 172 was not possible because of the small volumes of AL collected but sufficient numbers of cells were collected from sheep 173 to evaluate DC for MHC class II (DRα) and CD1 expression following primary antigenic challenge. These results are shown in Figure 6.1 and 6.2.

4.5.1. Primary antigenic challenge

There was no change in the proportion of cells expressing MHC class II or CD1, or in the intensity of expression of these molecules on day 1 following antigenic challenge, although on day 3 there was a small increase in the proportion

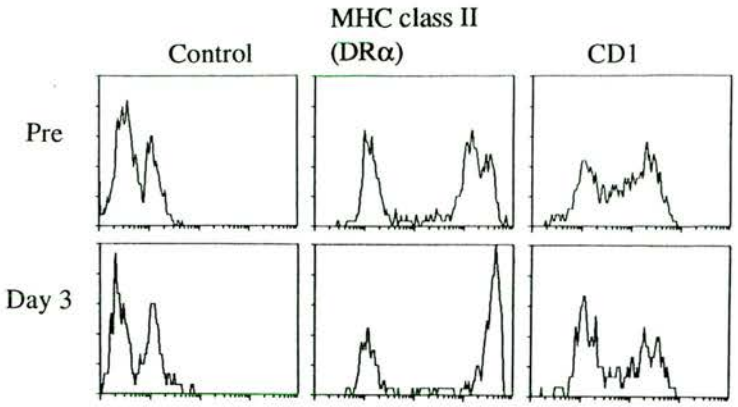
Figure 6.1 and 6.2. Phenotypic expression of DC in AL from the popliteal pseudoafferent lymphatic vessel at various time ponts following primary (6.1a) and secondary antigenic challenge (6.1b) of the TT joint.

Figure 6.1. Flow cytometry profiles of AL DC labelled with Mab to MHC class II antigens, CD1 and CD44, from sheep 173 (a) and sheep 139 (b) at various time points following antigenic challenge of the TT joint.

Figure 6.2. Graph to show the percentage values of AL DC positively-labelled with the above Mab obtained by flow cytometry. The arrow indicates the time of antigenic challenge of the joint.

Figure 6.1

a. Sheep 173



b. Sheep 139

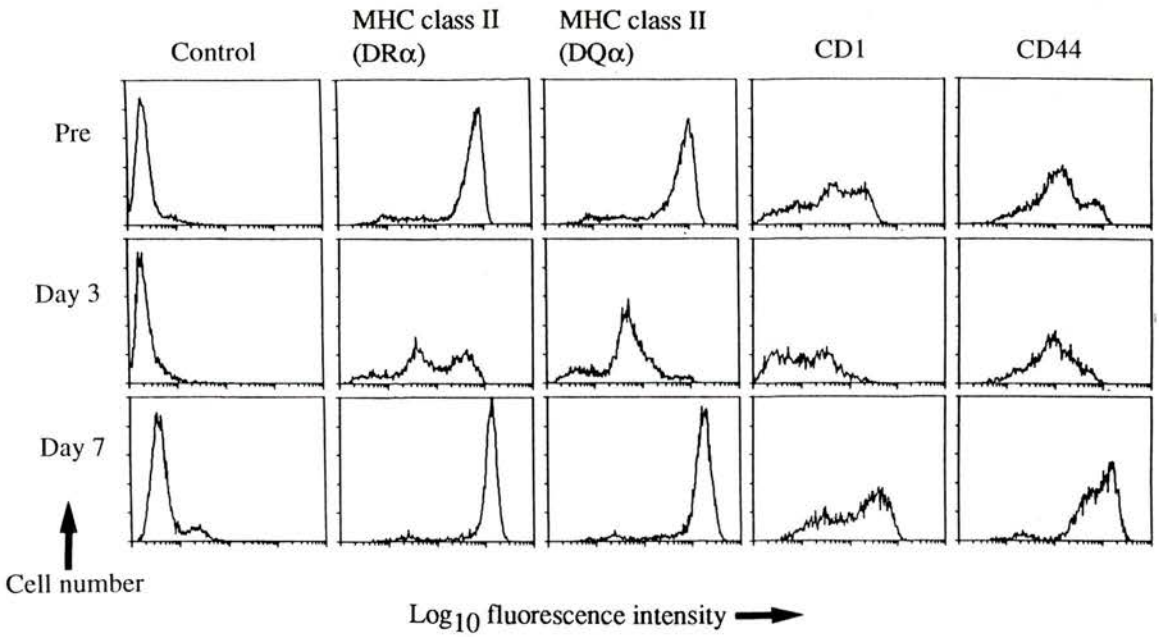
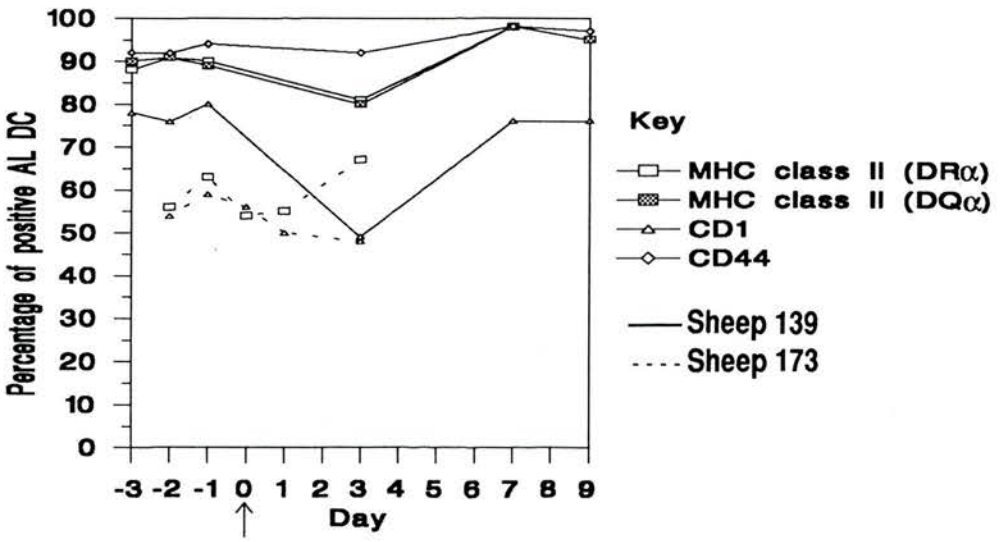


Figure 6.2



of cells expressing MHC class II and in the intensity of its expression (Figure 6.1a and 6.2).

4.5.2. Secondary antigenic challenge

The first time point following antigenic challenge that AL DC were evaluated was day 3 because of high levels of fluorescence of this cell population resulting from uptake of FITC-labelled OVA (see section 5). On day 3 post-antigenic challenge the percentage of cells positive for all the above antigens was reduced compared to pre-challenge levels, with the exception of CD44 (Figure 6.1b and 6.2). Additionally, their levels of expression were significantly reduced at this time although less markedly for CD44 than for the other molecules. At day 7 and day 9 the proportion of AL DC expressing these molecules were at pre-challenge levels but their intensity of expression was increased.

Similar conclusions apply to these results as were made in section 3.4.. The concentration of these cells was increased in AL following primary antigenic challenge (173) and their output in AL was increased following secondary antigenic challenge (139). This suggests that at least some of the DC collected were joint-derived. However, the changes in the intensity of expression of the molecules investigated affected virtually all of the DC. In addition, the reduced intensity of expression of MHC class II on DC on day 3 following secondary antigenic challenge followed by the increased intensity at day 7 corresponded to similar changes of MHC class II expression by T lymphocytes in AL from this sheep. In contrast, whilst the intensity of MHC class II expression was increased on DC on day 3 following primary antigenic challenge, the intensity of expression on T lymphocytes at this time was the same as pre-challenge levels (Figure 4a).

5. Appearance of FITC-labelled antigen on cells in AL

To monitor the appearance of antigen on cells in AL following its injection, OVA and HSA were FITC-labelled. At the time points detailed in section 4 a sample of the AL was washed 3 times in 2% BSA/Az and fixed in 0.5% paraformaldehyde. The fluorescence of the different cell populations was then determined by flow cytometric analysis by "live" gating on the different cell populations. The positivity of each population was compared to background levels of autofluorescence measured prior to antigenic challenge. For neutrophils, which were virtually absent from normal resting lymph, the background level of autofluorescence was made by reference to neutrophils from PB. The first time point that neutrophils were assessed was at 8 hours following antigenic challenge. The results obtained following primary (173) and secondary (139) are shown in Figure 7.1 and 7.2.

5.1. Primary antigenic challenge (sheep 173)

Only a small percentage of fluorescent DC, lymphocytes and neutrophils were present in the AL (Figure 7.1 and 7.2). The highest proportion of fluorescent DC was recorded at 4 hours post-challenge.

5.2. Secondary antigenic challenge (sheep 172 and 139)

The percentage of fluorescent AL DC increased markedly following antigenic challenge in sheep 139, peaking at 6 to 8 hours (Figure 7.1 and 7.2). For sheep 172 the maximum number of fluorescent DC was observed at 2 hours (48% DC fluorescent)(data not shown). Normal background autofluorescence levels were reached at 12 and 72 hours for sheep 172 and 139 respectively. In addition, there were more DC with a high level of fluorescence in the AL of sheep 139, compared to those from sheep 172.

A high proportion of the neutrophils were fluorescent in the AL of sheep 139 at 8 hours (Figure 7.1 and 7.2). This fell sharply at 12 hours but then increased at

Figure 7. Detection of antigen on different cell populations in AL from the popliteal pseudoafferent lymphatic vessel following its injection into the TT joint.

Fluoresceinated HSA (FITC-HSA) was injected into the TT joint of sheep 173 (primary immune challenge) and FITC-OVA was injected into the TT joint of sheep 139 (secondary immune challenge). The appearance of antigen on the cell populations in AL (DC, lymphocytes and neutrophils) was monitored by flow cytometric analysis of these populations at time intervals following antigenic challenge. The live gates used to evaluate these cell populations were shown in Chapter 2, Figure 3.1c.

Figure 7.1 shows flow cytometry profiles of each cell population at 4 or 8 hours following antigenic challenge. The dotted line in each graph shows the level of autofluorescence for that cell population obtained immediately prior to antigenic challenge.

Figure 7.2 shows the percentage values obtained by flow cytometry for each cell population from both sheep at various time points following antigenic challenge (0 hours) The percentage of each cell population fluorescent for antigen was determined by reference to the level of autofluorescence on each cell population prior to the injection of antigen. For neutrophils, which were not present to any great extent in AL prior to antigenic challenge, the background level of autofluorescence was determined from neutrophils from the PB.

Each cell population was monitored until the level of fluorescence returned to pre-challenge levels (or in the case of neutrophils until they were no longer detectable in the AL).

Figure 7.1

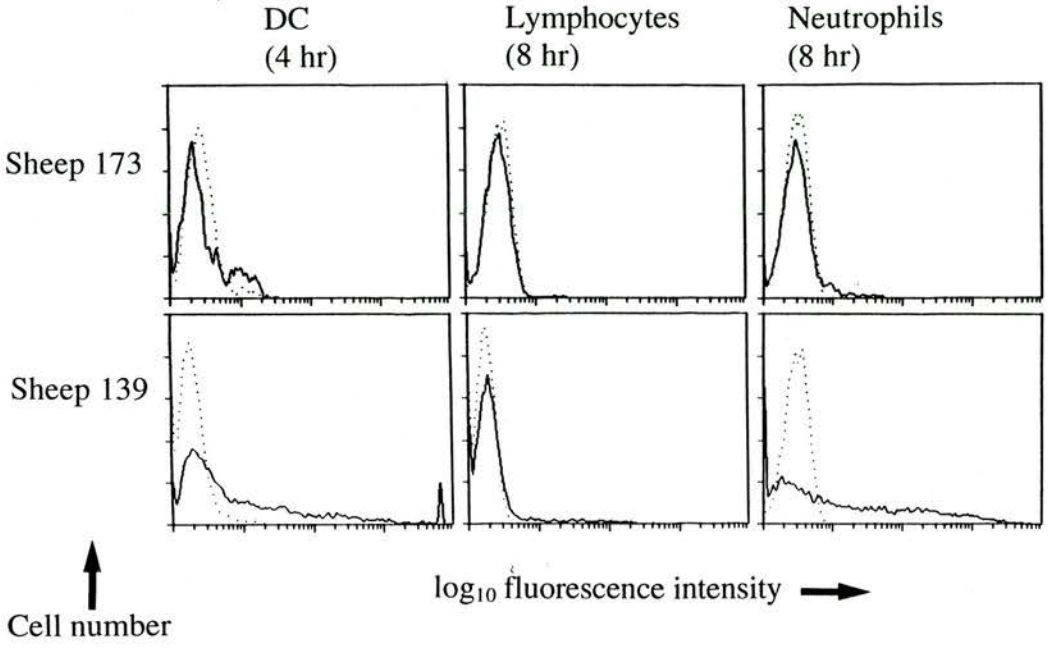
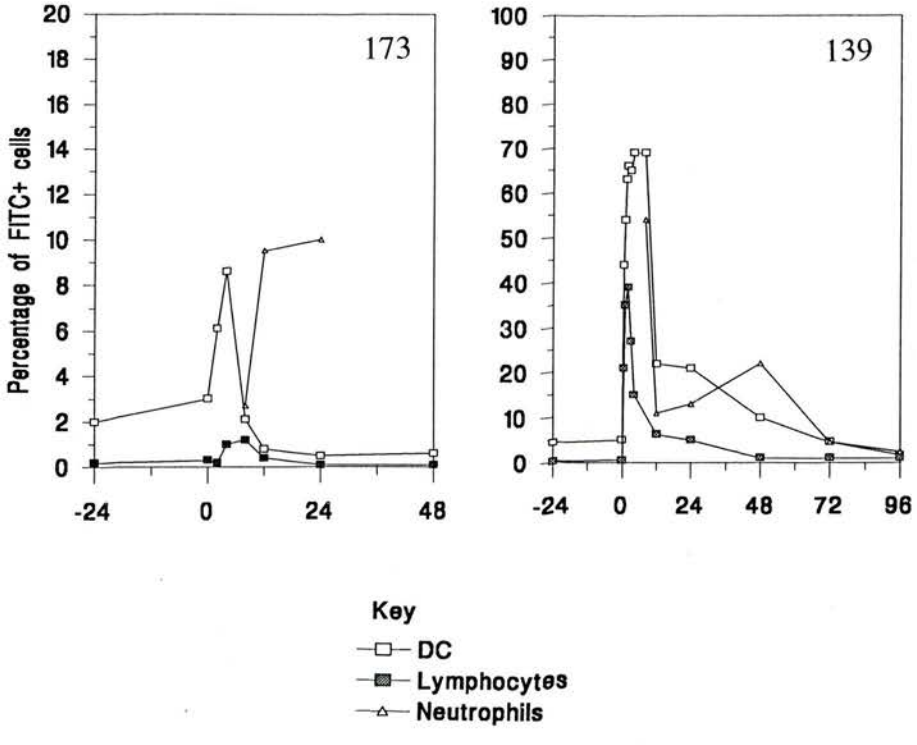


Figure 7.2



48 hours. The reasons for this were unclear. The proportion of fluorescent neutrophils in the AL of sheep 172 were much smaller by comparison (8% of cells were fluorescent at 12 hours)(data not shown). In both sheep, neutrophils comprised less than 10% of the cells in AL at 24 hours at which time fluorescence levels could not be accurately determined.

Up to 40% of the lymphocytes in the AL of sheep 139 were fluorescent at 2 hours. This decreased to background levels by 12 hours (Figure 7.1 and 7.2). Very few fluorescent lymphocytes (<2%) were detected in the AL of sheep 172 at any time point.

In these experiments labelled-antigen could have been acquired by cells either in the joint or in the collecting bottle. The high levels of fluorescence recorded immediately following antigenic challenge suggest the latter explanation, although it is possible that some of the antigen was acquired by these cells in the joint. Generally, a higher proportion of fluorescent cells for each cell population was recorded following secondary antigenic challenge, and the intensity of fluorescence was higher than following primary antigenic challenge. This suggests that following secondary antigenic challenge, antigen was probably acquired by cells in the form of IC.

6. PKH-2 labelling of AL DC to determine whether cells in SF can migrate and be detected in the AL

In order to determine whether cells can migrate from the SF into the afferent lymphatics an experiment was performed with *in vitro*-labelled AL DC, in sheep 139, approximately 3 weeks following antigenic challenge of the joint. AL from this sheep was collected over a 20 hour period and DC were isolated by centrifugation over metrizamide. These cells were then labelled with PKH-2 (Flow cytometric analysis of these cells confirmed a high uniform level of fluorescence, shown in Figure 8.1), resuspended in 0.75ml AL supernatant and injected into the

Figure 8. Demonstration of migration of DC from SF into the AL draining the joint.

Figure 8.1. AL was collected over a period of approximately 20 hours from sheep 139 and the DC isolated by centrifugation over metrizamide. These cells were then labelled with the fluorescent dye PKH-2. Confirmation of uptake of dye was provided by flow cytometric analysis (Figure 8.1). The dotted line on the profile indicates the level of autofluorescence of AL DC following incubation in diluent alone. This confirmed a uniformly high level of PKH-2 uptake. $4.8x10^5$ AL DC were injected into the TT joint and the AL from the popliteal pseudoafferent vessel was collected at regular intervals to determine the proportion of fluorescent DC at each time point.

Figure 8.2 shows flow cytometry profiles of AL DC collected immediately prior to, and at 18 hours following the intraarticular injection of labelled DC. 0.23% of the AL DC collected at 18 hours have a level of fluorescence that could be attributable to PKH-2 labelling.

Figure 8.3 shows the percentage values obtained from the flow cytometric analysis (shown in Figure 8.2) of AL DC with an intensity of fluorescence attributable to PKH-2, recovered at various time points following the injection of labelled DC into the joint.

Figure 8.1

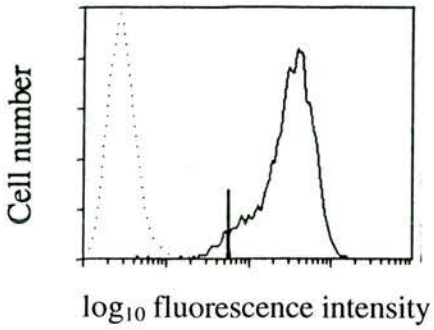


Figure 8.2

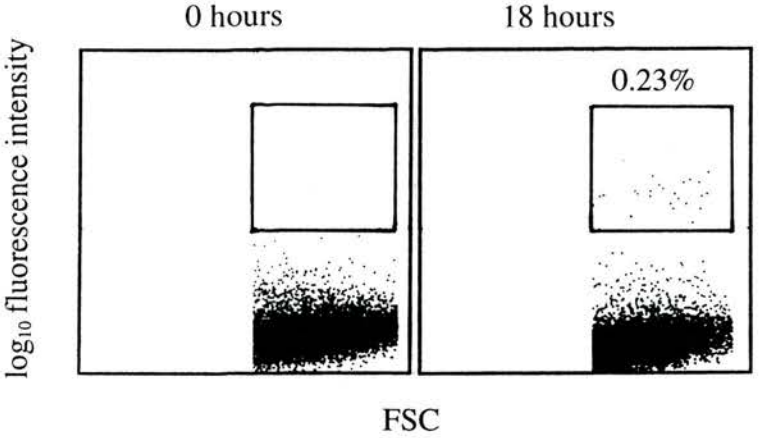
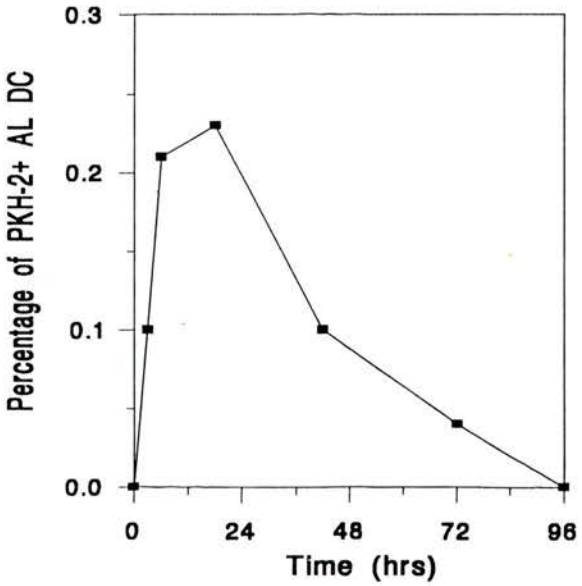


Figure 8.3



TT joint. A total of $4.8x10^5$ AL DC were injected into the joint space. The sheep at this time showed no residual lameness or periarticular soft tissue thickening from the previous antigenic challenge of the joint. Following collection, the cells in AL were washed 3 times in 2% BSA/Az. The presence of fluorescent DC in AL was then monitored by flow cytometric analysis of this cell population at different time points (3, 6, 18, 40, 72 and 96 hours following injection). In addition, 8 cytospin preparations were made at each time point and examined by fluorescence microscopy. The level of autofluorescence of DC was determined on 3 consecutive days prior to the experiment. On no occasion were cells detected, in either lymphocyte or DC "live" gates, with the intensity of fluorescence of PKH-2-labelled cells. The results of this experiment are shown in Figure 8.2 and 8.3.

Very few fluorescent DC were detected in AL. The highest proportion of fluorescent DC was 0.23% at 18 hours following injection (Figure 8.2). No fluorescent cells were detected within the lymphocyte "live" gate at any time and none were detected by fluorescence microscopy. At 96 hours, when fluorescent cells were no longer detectable in AL (Figure 8.3), SF was aspirated and the larger non-lymphoid population of cells evaluated for fluorescence by flow cytometric analysis and fluorescence microscopy. By flow cytometric analysis, only 0.18% of these cells had a level of fluorescence that could have been attributable to PKH-2 staining (data not shown). The total recovery of fluorescent cells was estimated to be $0.38x10^5$ which represented approximately 8% of the total number of DC injected into the joint.

The low recovery rate of labelled cells in this experiment can be interpreted in several ways. Some of the cells may not have survived the inflammatory response associated with their introduction into the joint, or these cells may have entered the synovial lining and not passed into the lymphatics. Also, there may have been a degree of chronic inflammation of the synovium from the initial antigenic challenge which may have influenced the survival or migration of these cells.

7. Changes in AL following induction of a flare reaction

To determine whether there are differences in the kinetics or phenotype of cells trafficking through a joint during a flare reaction compared to secondary antigenic challenge of a joint, 3 sheep had AIA (0.5mg OVA) generated in a TT joint 4 to 8 weeks before cannulation of the pseudoafferent lymphatic vessel. The surgical procedure was successful in 1 sheep (number 180). 5 days after surgery, 0.5 mg OVA was injected into the TT joint with chronic AIA. At this time, the sheep showed no residual lameness from the first injection of OVA but there was some mild periarticular soft tissue thickening of the joint. AL was collected daily for 7 days following antigenic challenge of the joint and its cellular composition analysed in a similar fashion to that described in section 4.

In addition, 6 weeks after induction of AIA in sheep 139, 0.5mg OVA was injected into the same TT joint. AL was collected on days 1, 2, and 5 following antigenic challenge. Clearly, the response of sheep 139 may have been modified by the chronic drainage of lymph from the distal limb and by the injection of labelled AL DC into the joint 10 days before induction of the flare reaction.

7.1. Volume and cellular composition of AL

The daily volume of AL in these sheep could not be accurately determined because of frequent blockage of the cannulae with fibrin clots.

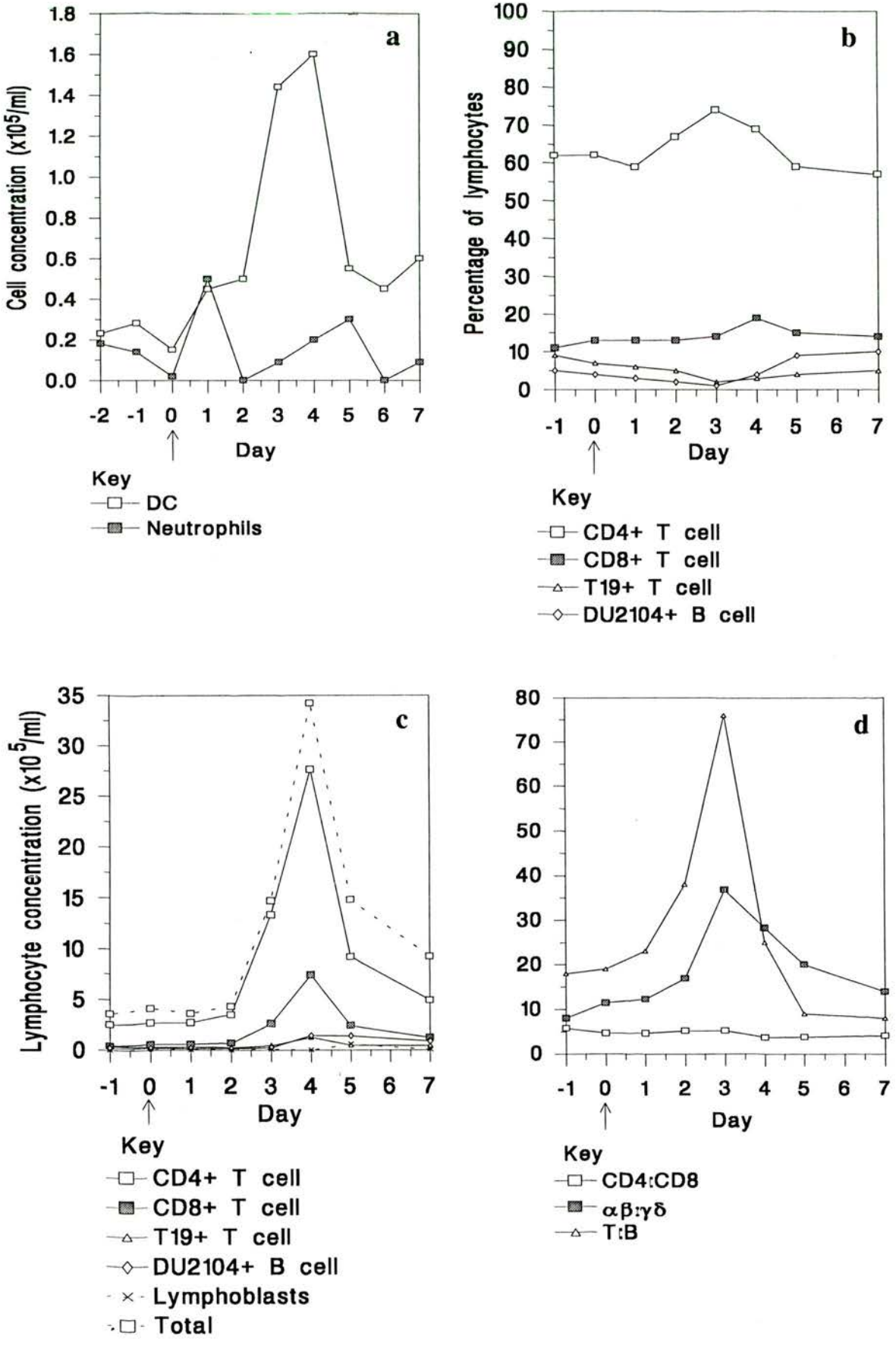
The changes in cellular composition and concentration of cells in AL from sheep 180 are shown in Figure 9. Increased numbers of neutrophils were present on day 1 following antigenic challenge (Figure 9a). However, as a proportion of cells in AL they represented 10 and 25% of its composition, in sheep 180 and 139 respectively. Neutrophil numbers had returned to normal by day 2 in both sheep. For sheep 139 these results were in contrast to the results previously obtained for secondary antigenic challenge of the same joint where at one point neutrophils comprised 80% of the cells in AL and their numbers had remained elevated for a longer duration.

Figure 9. Cellular composition of AL from the popliteal pseudoafferent lymphatic vessel following induction of a flare reaction in sheep 180.

AIA was generated in a TT joint of sheep 180 with 0.5mg OVA. Approximately 5 weeks later the popliteal pseudoafferent lymphatic duct in the same limb was cannulated and a flare reaction induced by injection of 0.5mg OVA into the same TT joint.

The concentrations of DC, neutrophils and lymphocytes in AL (a and c) were determined from the differential cell counts and the TNCC. The percentages of the B and T lymphocyte subsets (b) were obtained by flow cytometric analysis of the lymphocyte population using the live gate shown in Chapter 2, Figure 3.1c. The concentrations of the lymphocyte subsets were calculated from these values together with the differential and TNCC (c). The CD4:CD8, $\alpha\beta$:$\gamma\delta$ and T:B lymphocyte ratios (d) were calculated from the results shown in (b). The arrow indicates the time of antigenic challenge of the joint.

Figure 9


a
Cell concentration (x10⁵/ml)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-2 -1 0 1 2 3 4 5 6 7
Day
Key
DC
Neutrophils
b
Percentage of lymphocytes
100
90
80
70
60
50
40
30
20
10
0
-1 0 1 2 3 4 5 6 7
Day
Key
CD4+ T cell
CD8+ T cell
T19+ T cell
DU2104+ B cell
c
Lymphocyte concentration (x10⁵/ml)
35
30
25
20
15
10
5
0
-1 0 1 2 3 4 5 6 7
Day
Key
CD4+ T cell
CD8+ T cell
T19+ T cell
DU2104+ B cell
Lymphoblasts
Total
d
80
70
60
50
40
30
20
10
0
-1 0 1 2 3 4 5 6 7
Day
Key
CD4:CD8
αβ:γδ
T:B

In contrast to secondary antigenic challenge of the joint (section 4.2.2.), there were changes in the percentages of the B and T lymphocyte subsets in AL and large increases in the concentrations of these cells (shown for sheep 180 in Figure 9b and c). The peak concentration of lymphocytes was on day 4 in sheep 180. This change represented approximately a 10-fold increase over pre-challenge concentrations. In sheep 139, there were 3- and 5-fold increases in the concentration of lymphocytes on days 1 and 2 following antigenic challenge respectively, compared to pre-challenge levels. On day 5, the increase was approximately 7-fold. Small numbers of large basophilic lymphoblasts were present in the AL of both sheep. The highest proportions (4% of total lymphocytes in both sheep) and concentrations of these cells were present on day 5. No cells with the appearance of plasma cells were observed in the AL of either sheep.

In sheep 180, most of the increase in the concentration of lymphocytes was due to CD4+ and CD8+ T lymphocytes. The CD4:CD8 ratio remained relatively constant throughout the course of the experiment (Figure 9d). On days 5 and 7 the percentage of B lymphocytes was increased compared to pre-challenge levels (Figure 9b). This resulted in a lowered T:B ratio on these days (Figure 9d). Although flow cytometric analysis of lymphocytes in AL was only performed on days 1 and 5 following antigenic challenge in sheep 139, similar changes in these ratios compared to pre-challenge values were observed. In parallel with the increases in lymphocyte concentrations, there was a marked increase in the concentration of DC following antigenic challenge in both sheep (shown for sheep 180 in Figure 9a).

The above changes showed that cell trafficking through an inflamed joint during a flare reaction was different in character and magnitude compared to a secondary immune response of a joint that had not previously encountered antigen. The changes in the T:B and $\alpha\beta$:$\gamma\delta$ T cell ratios during the flare response suggested that either $\gamma\delta$ T cells and B cells entered the joint in significantly smaller numbers compared to CD4+ and CD8+ T cells or that they were selectively retained within

the joint tissues. Although these ratios were calculated for joint tissues on day 3 following antigenic challenge in Chapter 5, the proportions of these cells may be different during a flare response. Therefore, the 2 sheep that had AIA generated in a TT joint at the same time as sheep 180 had flare reactions induced in the these joints with 0.5mg OVA. SF was aspirated on day 3 and the proportions of B and T lymphocytes calculated following flow cytometric analysis in the manner previously described.

The αβ:γδ T cell ratios in SF from these sheep were 9.4 and 7.1 and the T:B ratios were 6.6 and 59. Compared to the peak αβ:γδ T cell ratio of 38 on day 3 in sheep 180 these results suggest that γδ T cells are retained in the joint during a flare response. Because of the variation in the T:B ratios from the SF of these 2 sheep similar conclusions could not be drawn for B cells.

7.2. Activation status, CAM and CD45RA expression of lymphocytes in AL

These were only evaluated for lymphocytes from the AL of sheep 180. The expression of MHC class II (pan reagent) was evaluated for the T lymphocyte subsets in the same manner described in section 3.3. The expression of CAM and CD45RA by lymphocytes was determined in the same manner as described in section 3.4. In addition, the expression of LFA-1 was also determined. These results are shown in Figures 10 and 11.

Prior to antigenic challenge the proportion of T lymphocytes expressing MHC class II and its intensity of expression were considerably higher than for previous sheep (shown for CD4+ T cells in Figure 10.1). It is possible that its expression was influenced by the effects of collecting lymph that was draining a site of chronic inflammation. Following the flare response there were minimal changes in the percentages of the T cell subsets expressing MHC class II (Figure 10.2) but the intensity of its expression fell progressively following antigenic challenge (shown for CD4+ T cells in Figure 10.1). The lowest level of MHC class II expression was observed on the day of peak lymphocyte concentration in AL (day 4)(Figure 10.1).

Figure 10.1 and 10.2. Expression of MHC class II by T lymphocytes in AL following induction of a flare reaction.

The expression of MHC class II (pan MHC class II reagent) by T lymphocyte subsets in AL was determined by double-labelling flow cytometric analysis.

Figure 10.1 shows flow cytometry profiles obtained by double-labelling with Mab to CD4 and MHC class II (pan reagent) at various time points following induction of the flare reaction. The dotted line on each profile is the level of autofluorescence of CD4+ T lymphocytyes obtained when the Mab to MHC class II was replaced with diluted normal rat serum (1:500).

Figure 10.2 shows the proportion of each T lymphocyte subset positively-labelled with Mab to MHC class II. These values were obtained by flow cytometric analysis. The arrow indicates the time of antigenic challenge.

Figure 10.1

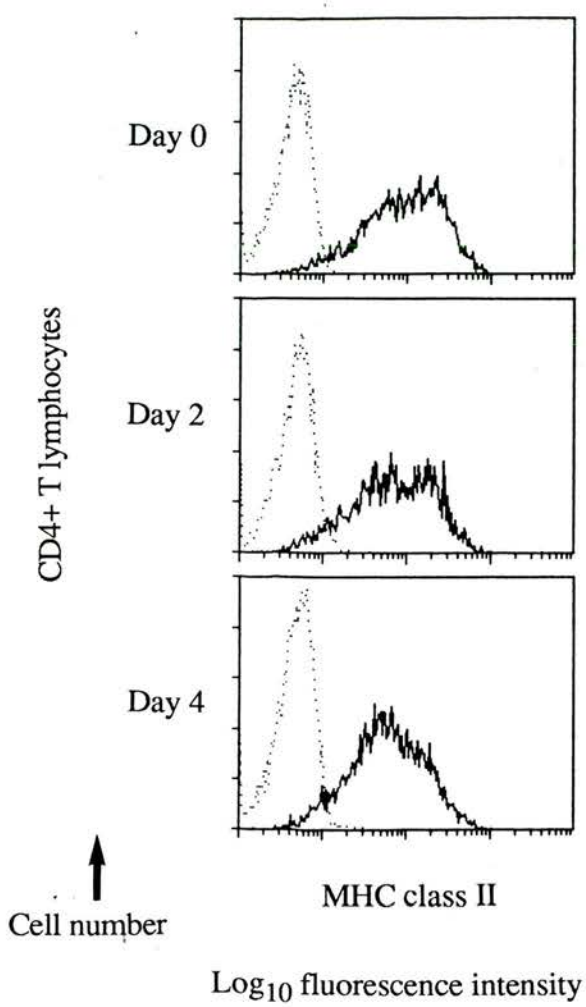


Figure 10.2

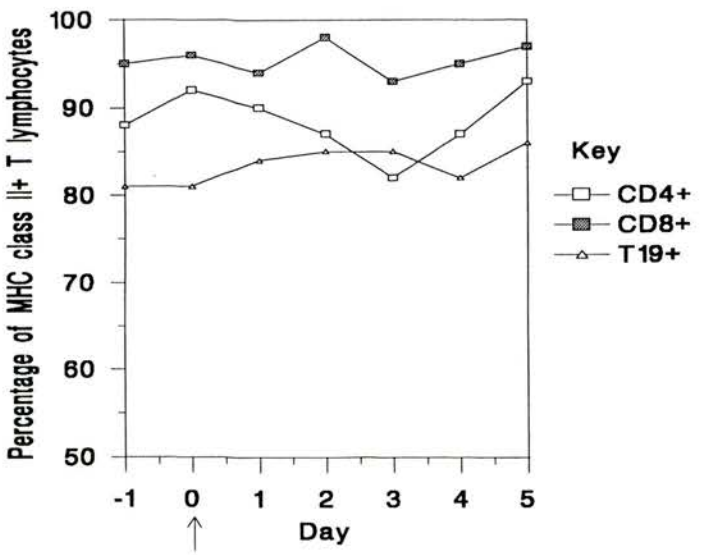


Figure 11.1 and 11.2. Expression of CAM and CD45RA by lymphocytes in AL from the popliteal pseudoafferent lymphatic duct following induction of a flare reaction in the TT joint.

Figure 11.1 Flow cytometry profiles of lymphocytes labelled with Mab to CD45RA, LFA1, L-selectin and CD44 at various time points following induction of the flare reaction.

Figure 11.2. Graph to show the percentage values obtained by flow cytometric analysis of lymphocytes for the above Mab. In addition the percentage values of lymphocytes positive for VLA6 and β-1 integrin are shown. Although there was minor fluctuation in the proportion of lymphocytes labelled with these two Mab, their intensity of expression did not change throughout the course of the experiment. The arrow indicates the time of antigenic challenge.

Figure 11.1

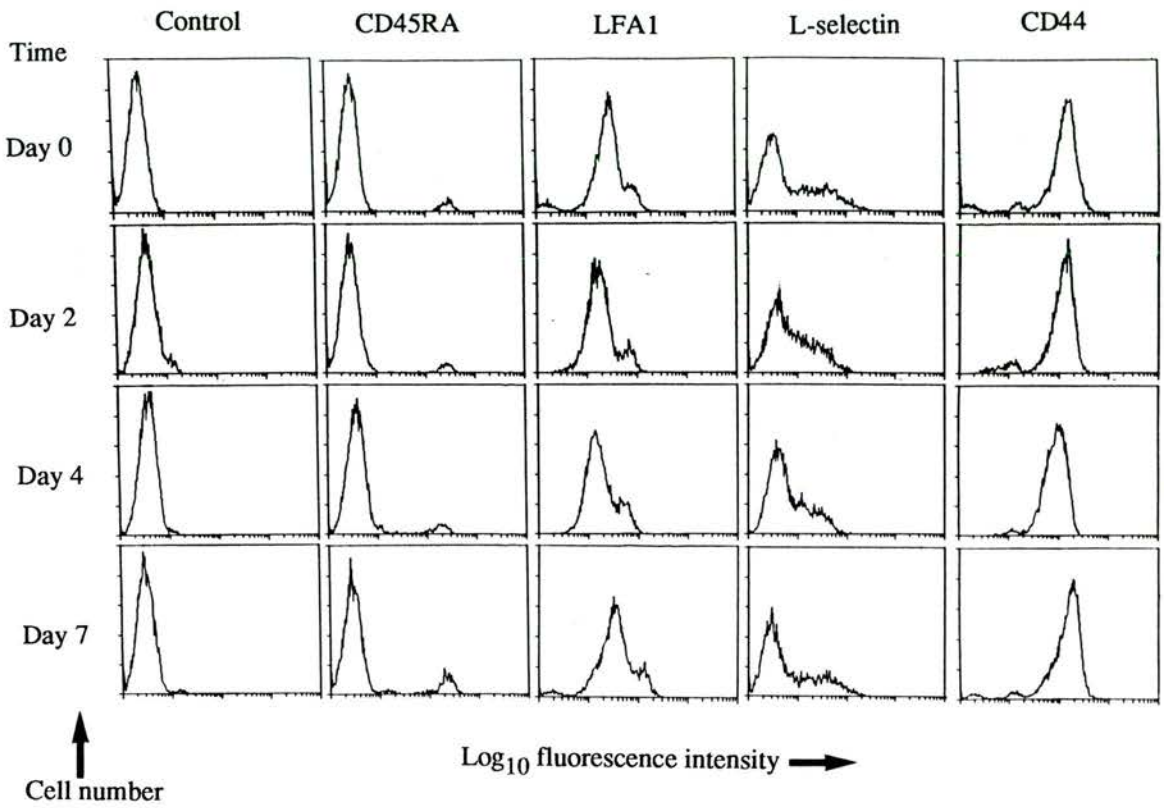
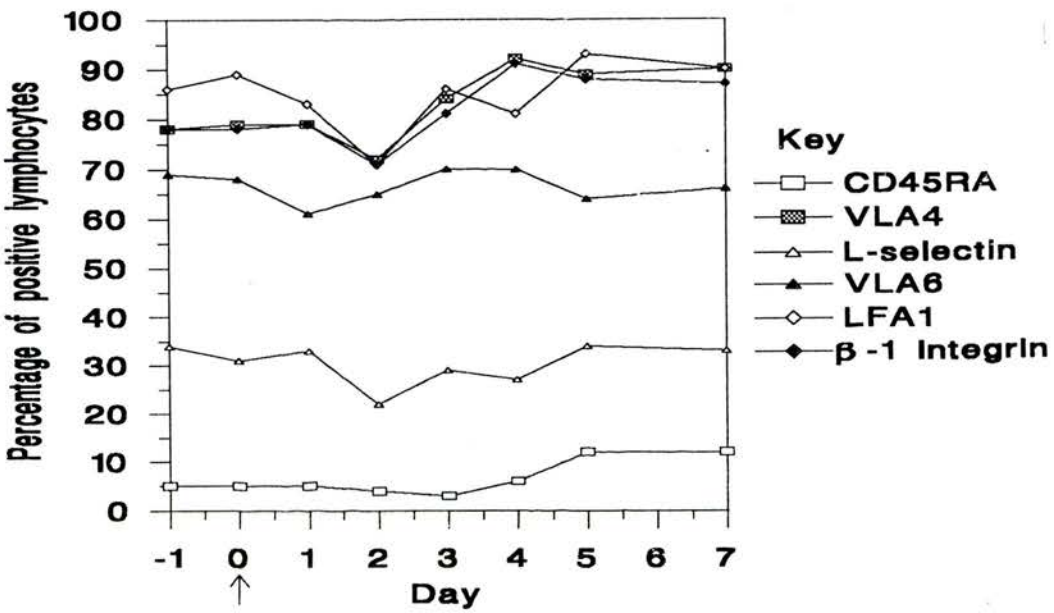


Figure 11.2



The intensity of expression of MHC class II on day 5 was identical to that of day 4. Insufficient numbers of cells on day 7 precluded analysis by double-labelling.

Only those CAM whose intensity of expression changed over time are show in Figure 11.1. The proportion of lymphocytes expressing the different CAM are shown in Figure 11.2. There was relatively little fluctuation in the proportion of lymphocytes expressing each CAM (Figure 11.2). The lowest level of expression of LFA1 and CD44 was recorded on day 4, corresponding to the peak lymphocyte concentration in AL. The expression of L-selectin also differed at different time points. Prior to antigenic challenge a small proportion of lymphocytes expressed high levels of this CAM ($>10^2$ fluorescence intensity) but on days 2 and 4 no high-expressing cells were present (Figure 11.1). However, by day 7 they had reappeared. These results were in marked contrast to those previously obtained following primary and secondary antigenic challenge of the joint (Figure 5a and b). The proportion of cells expressing CD45RA remained relatively constant until day 5 when an increase was recorded. This increase was most probably due to the increased percentage of B lymphocytes in AL at this time.

It was of interest that changes in the expression of some of these molecules eg LFA1 and L-selectin were different on day 2 following antigenic challenge compared to pre-challenge levels. At this time the concentration of lymphocytes in AL was the same as prior to antigenic challenge. It was not known what proportion of these cells were joint-derived prior to induction of the flare. It is possible that as a proportion of the cells in AL, joint-derived cells formed a higher proportion of the cells collected compared to the initial experiments. The conclusions made in section 4.4.2. may also apply to the interpretation of these results during the flare response.

7.3. Demonstration of OVA-specific B cells and cells containing OVA-antibody complexes in AL, synovium and SF

Several cytospin preparations of AL from sheep 180 were made each day for several days prior to and following antigenic challenge. Following cold acetone fixation these were stored at -70C. Biotinylated OVA and HSA were used in a similar fashion to that described in Chapter 5, Part II, section 2.3. to identify cells with receptors for OVA (OVA-specific B cells) or containing complexed OVA.

No positively-stained cells were observed on cytospin preparations made prior to, or for the first 3 days following antigenic challenge. On day 4, several positively-stained cells were observed although their morphology could not be determined with certainty. On day 5 when the maximum number of large basophilic lymphoblasts were counted, the largest number of positively-stained cells were observed (approximately 30 cells on the cytospin preparation). Although the size and morphology of these cells varied, many did appear to resemble large lymphoblasts (Figure 12a). It was not possible to determine the proportion of positively-labelled lymphoblasts because of difficulties in differentiating them from DC.

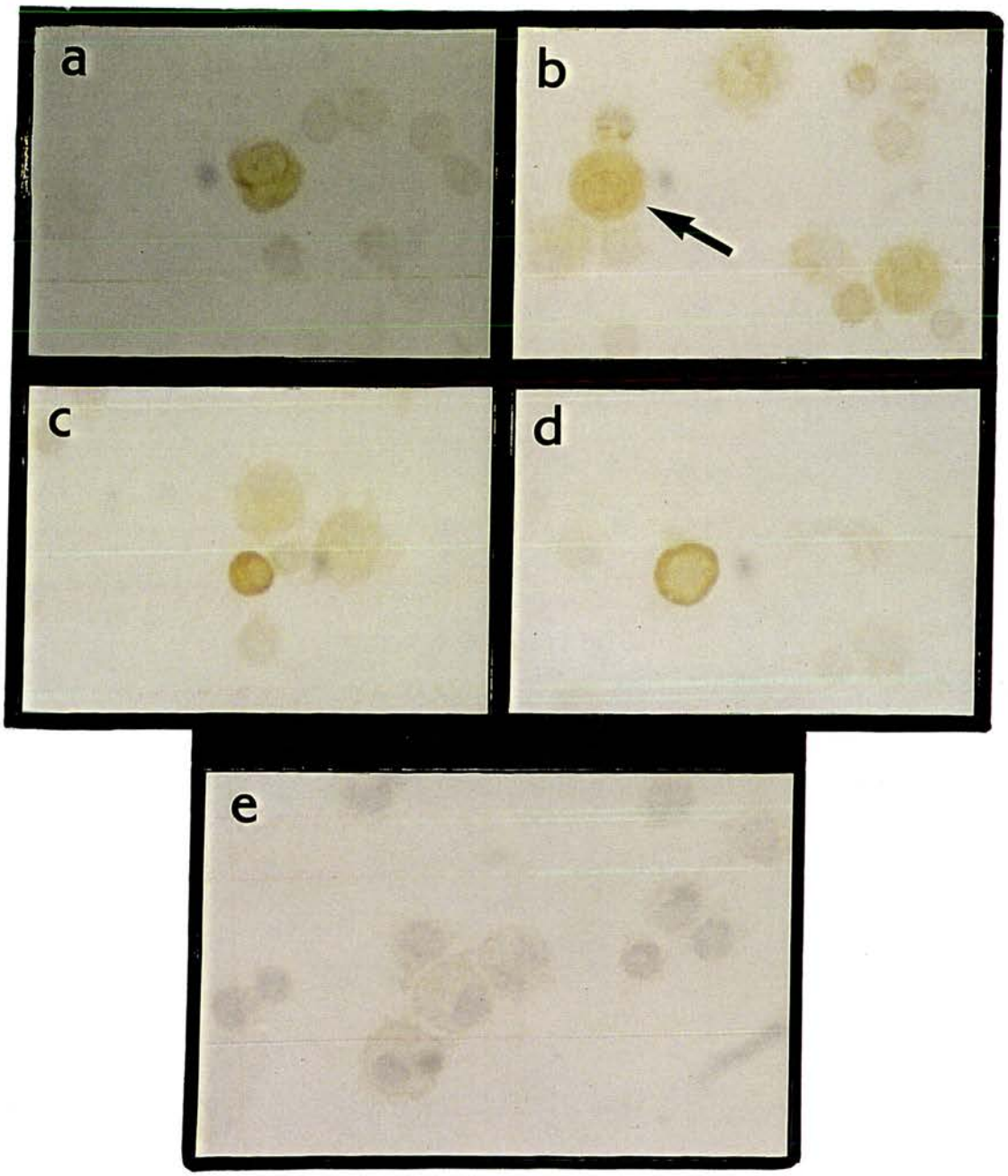
OVA was used on synovium from the antigen-challenged joints of sheep 139 and 180 in the same fashion as described in Chapter 5. The pattern of staining obtained with OVA was similar for both sheep and closely resembled that shown in Chapter 5 (Part II, Figure 3c). A large number of positively-stained cells of predominantly lymphoid morphology frequently clustered together to form large aggregates were present in synovium from both sheep. It would seem likely that some of these cells were OVA-specific B lymphocytes although double-labelling with B lymphocyte-specific Mab, which would have confirmed this, was not performed. Non-specific uptake of OVA by T lymphocytes would seem unlikely because no HSA+ cells were seen. However, the results obtained in section 5 suggested that some T lymphocytes could acquire OVA, presumably complexed

Figure 12. Identification of OVA-specific B lymphocytes and cells containing complexed OVA in AL and SF following induction of a flare reaction in sheep 180.

Cytospin preparations of AL from the popliteal pseudoafferent lymphatic duct were made daily before and after the induction of a flare reaction in the TT joint. Additionally, cytospin preparations of SF were made following euthanasia of the sheep on day 8 following induction of this reaction. Cells capable of binding OVA (OVA-specific B cells and cells containing OVA-antibody complexes in antibody excess such as macrophages and DC) were identified by incubating slides in dilutions of bOVA followed by peroxidase-labelled streptavidin and DAB solution. As a control, cytospin preparations were incubated in bHSA. Positively-labelled cells were identified in AL (a) and SF (b, c, d).

(a) A positively-labelled cell in AL on day 5 following induction of the flare reaction, with the morphology of a large basophilic lymphoblast. Positively-labelled cells in SF were identified with the morphology of synoviocytes (b, indicated with an arrow) and lymphoid cells (c and d). A cytospin of SF incubated in HSA is shown in (e).

Figure 12



antibody, and the possibility therefore exists that some of the positively-stained cells in synovium were T lymphocytes.

SF from the antigen-challenged joint of sheep 180 was aspirated following euthanasia on day 8. Following centrifugation over metrizamide to remove the neutrophils (75% of the cells in SF), cytospin preparations were made. These were investigated for OVA-positive cells in the same same way as for AL. Large numbers of positively-stained cells were present (Figure 12b, c, d). The morphology of these cells and their staining intensity varied considerably. Generally the most weakly stained cells had the appearance of synoviocytes (Figure 12b). This was in contrast to the staining pattern obtained for synovium where no positively-stained SLC were observed. A moderate number of small positively-stained cells were observed with a lymphoid morphology (Figure 12c and d) and some phagocytic cells were seen with what appeared to be fragments of positively stained cells within their cytoplasm.

No cells stained positively with HSA on cytospins of AL and SF (Figure 12e), or on sections of synovium.

7.4. Histological and immunohistological appearance of synovium and SF analysis following a flare reaction

Synovium from sheep 139 and 180 was obtained at post mortem 6 and 8 days respectively following induction of the flare response. In both sheep synovium was characterised by moderate SLC hyperplasia and very large numbers of lymphoid cells that formed a continuous broad band beneath the SLC (Very similar in appearance to that shown in Chapter 5, Figure 5b). The numbers of these cells greatly exceeded the numbers present at any time point following initial induction of AIA. In sheep 139, only relatively small numbers of plasma cells were present compared to the number of small lymphocytes, whereas in sheep 180 larger numbers of plasma cells were present although these were still fewer in number than the small lymphocytes.

Immunohistology was performed on synovium from the antigen-challenged joints of both sheep with Mab to B and T lymphocyte subsets. Large numbers of CD4+ and CD8+ T lymphocytes were present in the synovium from both sheep. Although not quantitated, CD4+>CD8+ T lymphocytes in sheep 139 and CD8+>CD4+ T lymphocytes in sheep 180. Moderate numbers of DU2104+ B cells were present and only occasional γδ T lymphocytes were observed in tissues from both sheep.

SF from both antigen-challenged joints were analysed. The TNCC were 8 and $16x10^6$/ml for sheep 139 and 180 respectively. In both cases 60 to 70% of the cells were neutrophils, the remainder being lymphocytes and synoviocytes. Interestingly, large basophilic lymphoblasts that had been identified in AL were not seen in SF from either animal.

The proportions of B and T lymphocyte subsets in SF was determined by flow cytometric analysis. From these were calculated the CD4:CD8, αβ:γδ and T:B lymphocyte ratios. These ratios reflected the qualitative observations made from synovium ie CD8+>CD4+ T cells and the αβ:γδ T cell ratio was high (approximately 30) in the SF from sheep 180. The T:B lymphocyte ratio in SF from sheep 180 was 17. This was considerably higher than that found in AL on the last day of lymph collection (T:B ratio of 8) which suggested that DU2104+ B cells may have either lost their expression of this molecule on passing into SF or that they preferentially trafficked through the synovium with relatively few cells entering the SF compared to T cells. A similar observation was made when these ratios were compared in the different compartments from sheep 139.

Summary of findings

1. Primary and secondary antigenic challenge of the TT joint resulted in significant numbers of neutrophils appearing in the AL. There were also moderate increases in the numbers of lymphocytes and DC following antigenic challenge.

2. Changes in the expression of MHC class II and a range of CAM by lymphocytes and DC in AL were detected following antigenic challenge of the joint. Following primary antigenic challenge there was little change in the expression of these molecules by lymphocytes or DC. Following secondary antigenic challenge, the expression of these molecules was initially reduced and then increased to a level higher than that present prior to antigenic challenge.

3. Following primary antigenic challenge, using a fluoresceinated antigen, only a small proportion of the DC and neutrophils in AL were found to have taken up antigen. Following secondary antigenic challenge of the joint, using a fluoresceinated antigen, all cell types in AL were found to have taken up antigen.

4. Injection of PKH-2-labelled DC into the TT joint and monitoring the AL for their appearance confirmed that these cells could migrate from SF into the afferent lymphatics draining the joint.

5. Following induction of a flare reaction, large increases in the concentrations of lymphocytes and DC were present in the AL, which peaked at day 4 following antigenic challenge. Most of the increase in the lymphocyte concentrations was due to CD4+ and CD8+ T lymphocytes. Changes in the expression of activation and CAM by lymphocytes in AL were detected following induction of the flare reaction. Immunocytochemistry performed on synovium and cytospin preparations of SF and AL confirmed the presence of cells containing OVA-antibody complexes, or with receptors for OVA (B lymphocytes) in all 3 compartments.

Discussion

The results shown in this chapter demonstrate that it is possible to study the trafficking of cells through an inflamed joint using a popliteal pseudoafferent cannulation system. The early appearance of neutrophils in AL has previously been demonstrated in AL draining skin following the application of irritants and the subcutaneous injection of bacteria in sheep (Hall and Morris 1962, Colditz *et al* 1988). Although most neutrophils are arrested in the draining lymph node (Colditz

et al 1988) some may enter the efferent lymphatics (Hay 1979). The changes observed in the concentration and output of lymphocytes during the early stages of AIA were relatively mild. The timing of the peak output of lymphocytes on day 4 in the antigen-primed sheep (139) is similar to that observed following antigenic challenge of a lymph node in primed sheep (MacKay *et al* 1992a) although the magnitude of the increase is considerably less.

The most significant increases in lymphocyte concentration in AL were observed following a flare reaction. These increases compared to the mild changes observed following secondary antigenic challenge of a non-inflamed joint may have resulted from an increase in the number of HEV-like blood vessels in chronically inflamed synovium (Freemont *et al* 1983) together with altered expression of endothelial CAM. In sheep 180, most of the increased lymphocyte traffic through the joint was due to CD4+ and CD8+ T cells. The changes in the proportions of the B and T lymphocyte subsets and the time course over which they took place was very similar to that described for efferent lymph following secondary antigenic challenge of a lymph node (MacKay *et al* 1992b). Interestingly the lymphocyte concentrations in AL did not increase until day 3 post-antigenic challenge, unlike the results obtained with the other sheep where increased lymphocyte concentrations were apparant on day 1 following antigenic challenge. This delay in the appearance of increased numbers of lymphocytes during the flare response may have been due to a cell shut-down phenomenon, similar to that observed following secondary challenge of lymph nodes (Hopkins *et al* 1981a, b, McConnell *et al* 1981). The factors thought to be responsible for this phenomenon in lymph nodes, prostaglandins and complement components, are generated in the joint during AIA (Cooke and Jasin 1972, Henderson and Higgs 1987) and the presence of underlying chronic synovial inflammation may make synovium behave in a similar fashion to lymph node. The significant increases in the $\alpha\beta$:$\gamma\delta$ and T:B lymphocyte ratios from day 2 to day 4 of the flare reaction in sheep 180 showed that either B cells and $\gamma\delta$ T cells were locally retained in joint tissue over this period or that they

were not recruited into the joint. The moderate numbers of γδ T cells in the SF of the 2 sheep with flare responses suggested the former for this cell type. The presence of γδ T cells in resting AL shows that these cells do recirculate. It is of interest to speculate that during inflammatory responses in joints, a greater proportion of these cells may be retained or apoptose within the joint. The latter possibility is supported by evidence from severe combined immunodeficient mice transgenic for rearranged γ and δ genes (Spaner *et al* 1993). The majority of activated γδ T cells in these mice rapidly apoptose following exposure to antigen. However, in sheep 139 on day 1 of the flare response the proportion of these cells in AL relative to the αβ T cells was increased, unlike sheep 180 at the same time point. Interestingly, the proportion of neutrophils in the AL of sheep 139 was higher at this time compared to sheep 180. If γδ T cells are part of a first line of defense mechanism it is possible that there are similarities in either the chemoattractants responsible for the recruitment of γδ T cells and neutrophils and/or that their endothelial CAM requirements are similar. In this respect, P-selectin has been shown to support shear-dependent rolling of neutrophils (Bevilacqua and Nelson 1993) and bovine γδ T cells (Jutila *et al* 1994) on endothelial cells.

The precise identity of the large basophilic lymphoblasts in AL has been a subject of considerable debate (Hall *et al* 1967) although it would seem likely that they are immature B cell blasts (Hall and Morris 1962, Smith *et al* 1970). The proportion of these cells in AL from all sheep was low although the proportion derived from joint tissues may have been higher than calculated because of the dilution effect of lymphocytes draining other non-inflamed tissues. The slight delay in their appearance in AL compared to the peak total concentration of lymphocytes is similar to the observations of EL following secondary antigenic challenge of lymph node (Hay *et al* 1974, MacKay *et al* 1992a). The relatively small proportion of blasts in the AL from sheep 180 was a little suprising given the large increase in cell trafficking through the joint and the likely presence of antigen-specific T and B

cells together with large numbers of cells capable of efficiently presenting antigen, *in vitro* at least. In this respect the nature of the response of joint tissue to antigenic challenge would appear to be similar to that of skin (Hay 1979) but different from that of lymph node during secondary immune responses where these cells may represent up to 50% of the cells in EL (Hall *et al* 1967). This suggests that blastogenic responses of lymphocytes are down-regulated during inflammatory responses in joints. This could be mediated by prostaglandins (Phipps *et al* 1991), complement components (reviewed in Erdei *et al* 1991) or inhibitors of cytokines such as IL1 (Henderson *et al* 1988) or IL2 (Miossec *et al* 1987). The virtual absence of these blast cells in SF suggests that they preferentially migrate into AL rather than SF. Some cells in AL with a large lymphoblast morphology did stain positively with OVA demonstrating that some of these cells were specific for the inciting antigen.

Assessing the significance of changes in the expression of activation and CAM on T lymphocytes and DC during the progression of AIA proved more problematic. Altered expression of some of these molecules was apparant even when the cell concentrations in AL had returned to pre-challenge levels suggesting that soluble factors from the inflamed joint may have influenced the expression of these molecules on cells during the collection period. This could be addressed by having shorter collection periods although the volumes of AL collected over 16 hours in some sheep were small. The expression of CAM by lymphocytes in AL following secondary immune responses were generally lower than pre-challenge levels for several days, after which time the levels were often higher than pre-challenge levels. A similar pattern of observations were made from SF T lymphocytes at early and late time points following the generation of AIA (Chapter 5). However, there were several differences in the phenotypic expression of lymphocytes in the two compartments. The proportion of lymphocytes in AL expressing high levels of L-selectin increased following primary and secondary antigenic challenge (sheep 172 and 173) but T lymphocytes in SF always expressed

low levels of this molecule (Chapter 5). Additionally, only low levels of IL2r expression were detected on CD8+ T lymphocytes from AL of these sheep whereas up to 30% of these cells in SF expressed this molecule. These differences could have been due to activation phenomena operating locally in SF or preferential migration pathways of populations of lymphocytes with these phenotypes. Also, it is possible that lymphocytes that enter SF are retained for longer periods of time within this enviroment compared to lymphocytes that do not enter SF but that traffic through synovium into the afferent lymphatic vessels.

Memory T cells draining different organs have been shown to have a characteristic expression of activation and CAM (MacKay *et al* 1992b). The results shown here and in Chapter 5 show that the expression of these molecules by lymphocytes in SF and AL may differ at different time points during inflammatory responses in joints despite these cells maintaining a memory phenotype. The functional significance of this variation in expression of these molecules is unclear. The reduced expression of these and probably other molecules early in the inflammatory response may down-regulate antigen-specific responses and possibily reduce the likelihood of immune responses to host tissue components.

It is possible that cytokines or other inflammatory mediators derived from the joint may have been responsible for some of the phenotypic changes to both lymphocytes and DC in AL following antigenic challenge of the joint. IL1 has been detected in AL draining normal skin in man (Plachta *et al* 1988), and prostaglandins and PLA2 have been detected in the AL of sheep draining chronic inflammatory sites (reviewed in Chapter 1 section 9). The potent immunomodulatory role of prostaglandins has been previously commented upon. Another factor that may influence the surface expression of molecules on DC are IC. IC uptake by DC *in vitro* and *in vivo* has been shown to result in the rapid loss or reduced expression of a variety of cell surface molecules (Coughlan 1994). Following secondary antigenic challenge of skin the cell surface expression of many activation and adhesion molecules by DC in AL draining from these sites was

reduced for a period of 1 to 8 hours after which time their level of expression returned to normal or was increased (Coughlan 1994). Hopkins *et al* (1989) monitored the expression of MHC class II and CD1 antigens on AL DC for up to 8 days following secondary antigenic challenge of skin and showed that the expression of these antigens was increased between days 3 and 6. The initial reduction in the expression of cell surface molecules is thought to be a consequence of engagement of FcγR by IC (Coughlan 1994), which have previously been shown to be expressed by these cells (Harkiss *et al* 1990). The subsequent increase in the expression of MHC class II antigens has been shown to be associated with an enhanced antigen-presenting capacity of these cells (Hopkins *et al* 1989). The differences in the time course of these events compared to antigenic challenge of the joint (sheep 139) may reflect differences in the dose of antigen used (500μg OVA in the joint versus 50μg in the skin) or in antigen retention at these sites. Prolonged retention of antigen in the joint compared to skin was suggested by the detection of labelled antigen on DC in AL 48 to 72 hours following its injection intraarticularly whereas it was not detected on DC draining the skin 6 hours following antigen administration, even when 2mg of OVA was injected intradermally (Harkiss *et al* 1990).

The concentration or output of DC in AL was generally increased following antigenic challenge of the joint. The increase following induction of a flare response was the most dramatic. Although DC were not formally identified in synovium in Chapter 5, increased numbers of these cells have been identified in synovium at an early stage following the generation of AIA in rats (Verschure *et al* 1989) and in mice with CIA (Holmdahl *et al* 1991). The results described in this Chapter suggest that some of these cells may pass quite rapidly through synovium before appearing in the AL. Alternatively, DC resident in synovium may be stimulated to migrate to the draining lymph node. For skin it has been shown that intradermal injections of TNF-α cause migration of LC to the draining lymph node in a dose-dependent fashion (Cumberbatch and Kimber 1992). Although it has not

been demonstrated that this cytokine is produced in joints during AIA it would seem likely that elevated levels of this cytokine are present.

An attempt was made to address the question of whether DC in SF are capable of leaving the joint, via the lymphatics, with labelled AL DC. Although the proportion of recovered cells was very small, the results did suggest that AL DC were capable of migrating from SF into AL. This experiment was not entirely physiological because it is likely that most of the DC were skin-derived which may have influenced their capacity to migrate from a different enviroment. The factors responsible for cells migrating between SF and the synovial lining are poorly understood but presumably involve the regulation of expression of molecules responsible for adherence of cells to components of extracellular matrix such as collagen and fibronectin. The only CAM known to be differentially expressed on T cells in synovium compared to SF is $\alpha 4\beta 7$ (Lazarovits and Karsh 1993) which acts as a receptor for VCAM-1 and fibronectin.

The detection of FITC-labelled antigen on a variety of cell types in AL following intraarticular injection may have resulted from antigen uptake by these cells in the joint or in the collecting bottle. Although it was not possible to say with certainty where the antigen was acquired in these experiments, these results together with those from the cell labelling experiment with AL DC suggest that DC in SF may be capable of transporting antigen to the draining lymph node. B lymphocytes could acquire antigen via their antigen-specific receptors or FcγR if the antigen was complexed with antibody. T cells are also capable of capturing antigen particularly at high concentration (Lanzavecchia 1990) or via FcγR following activation (Sandor and Lynch 1993), which would allow them to acquire antigen in the form of IC. These results suggested that antigen could be transported to the draining lymph node associated with a number of different cell types.

The large numbers of cells that stained positively for OVA in the synovium from sheep 139 and 180 and their relative paucity in the AL of sheep 180

suggested that OVA-specific B cells were retained locally within the joint. The presence of OVA-specific T lymphocytes in SF at day 3 following a flare reaction was shown previously in Chapter 5. Whether there is specific retention of these T cells in joint tissues in a manner similar to that described for lymph node during the early phase of secondary immune responses (Hay *et al* 1974) remains to be established. Large numbers of non-lymphoid cells in SF from sheep 180 stained positively with OVA. This was most probably due to these cells containing OVA-antibody complexes and may explain why high levels of background proliferation were obtained in the T lymphocyte proliferation assays described in Chapter 5.

In the majority of sheep in this study the α–OVA IgG antibody titres in AL were moderately elevated at 24 to 48 hours following antigenic challenge (data not shown). After this time the antibody titres were at pre-challenge levels. These are consistent with the findings of Thorpe *et al* (1992) who showed a similar increase in the level of antigen-specific antibody in AL draining joints following generation of CICIA in sheep. Similar to the results obtained in Chapter 5, higher levels of α–OVA IgG were present in SF from the antigen-challenged joint compared to the contralateral joint in all sheep described in this Chapter. The antibody titres in SF from the antigen-challenged joint were always higher than in the AL draining the joint suggesting that locally synthesised Ig that drains into the lymphatics of the joint is diluted by AL from other tissues.

The frequent problems encountered with blockage of cannulae by fibrin clots contributed to the difficulty of the experimental procedures described in this Chapter. Clearly if fibrin clots were to form in lymphatics in the absence of cannulae, this might contribute to the accumulation of cells in joints by preventing their natural route of exit from these tissues.

CHAPTER 7

Final Discussion

1. Introduction

The characterisation of synovial tissues and synovial immune responses in humans with inflammatory arthropathies and animal models of arthritis has significantly improved our understanding of the pathogenesis of inflammatory joint disease. Despite the ease with which synovial tissues can be obtained from sheep, the high prevalence of naturally-occuring inflammatory joint disease that affects this species (reviewed in Chapter 1) and the opportunites that are available to study aspects of the dynamics of synovial inflammation that can only be investigated in an animal of sufficient size to permit lymphatic cannulation studies to be performed, inflammatory joint disease in sheep has rarely been investigated. Hence the aims of this thesis were to characterise synovial tissues from normal sheep and from animals with natural and experimental arthropathies. An additional aim was to assess the suitability of this species as a host for investigating the dynamic aspects of synovial inflammation using lymphatic cannulation techniques.

2. Arthritis associated with MVV infection

Arthritis associated with retroviral infections in ruminants are naturally-occuring inflammatory arthropathies whose pathogenesis are of relevance to the understanding of similar disease processes in other species infected with these viruses, including humans. The disease process in ruminants may be somewhat easier to dissect compared to that of humans infected with HIV because of the more restricted range of cell types capable of supporting productive infection (Gendelmann *et al* 1985, Levy 1993) and because cofactors are thought to be less important in the pathogenesis of ruminant lentiviral infections compared to infection with HIV in humans (Blacklaws *et al* 1994a). The results shown in Chapter 4 together with observations of synovium obtained from humans infected with HIV (Dalton *et al* 1990) suggest that significant histological or immunopathological changes may be present in synovium from some joints of these species in the absence of overt clinical joint disease. Although MVV has been

identified by *in situ* hybridisation in histologically normal synovium from the joints of sheep (Brodie *et al* 1995), this method of detection may underestimate the numbers of infected cells in these tissues. The recent use of PCR-*in situ* hybridisation techniques in tissues from humans infected with HIV has demonstrated that a much larger number of cells are infected with this virus than was previously realised (Embretson *et al* 1993, Patterson *et al* 1993). Although this technique has been used to determine the number of infected cells in the lungs of sheep experimentally-infected with MVV (Staskus *et al* 1991) it has not been used on tissues from naturally-infected sheep. Although these studies would provide useful information on the virus load within tissues, they would give little indication of the dynamics of virus infection *in vivo*. Recent studies of the kinetics of virus and CD4+ T lymphocyte production and clearance in humans infected with HIV indicate that maintenance of steady-state levels of infection is a balanced, highly dynamic process (Wei *et al* 1995). The same may also be true of the virus load within individual organs or tissues. Although very little is known of the dynamics of ruminant lentiviral infections *in vivo*, an increase in the severity of the chronic inflammatory changes in target organs has been found to be associated with increased virus load within these tissues (Brodie *et al* 1992). Although the relationship between the presence of virus and severity of inflammation in these tissues is unclear, an imbalance between virus production and clearance resulting in an increased viral load may be an important feature of the development of lesions. An important feature of this concept is an understanding of cell turnover in tissues because alterations in the dynamics of cell trafficking may have a profound influence on the accumulation or clearance of infected cells. Although the main cell type capable of supporting productive infection with MVV is thought to be the macrophage (Gendelmann *et al* 1985, Brodie *et al* 1995), there is some evidence that DC may also be infected (Gorrell *et al* 1992). Some information pertaining to the dynamics of trafficking of DC through inflamed joints was obtained in Chapter

6 of this thesis. A fuller understanding of this process for DC and macrophages is of importance for understanding the dynamics of virus load in joint tissues.

One explanation for the relative ease with which chronic arthritis can be established with this virus and with CAEV in very young animals compared to adults may relate to an increased turnover of cells in the synovial lining that occurs as a normal feature of development or secondary to inflammatory events that take place during the early neonatal period (Chapter 3). The expression of FcγR by cells in the synovial lining at an early stage of development may also serve to enhance infection of these cells via antibody-mediated uptake of virus (Jolly *et al* 1989).

With the discovery that CD4+ T lymphocytes in humans and mice can be divided into different subsets on the basis of their expression of cytokines following stimulation *in vitro* (Seder and Paul 1994, Mosmann *et al* 1986), and the importance of these subsets in determining the outcome of infections with some infectious agents such as Leishmania (Scott *et al* 1988), there is considerable interest in the role they play in the progression of disease of retroviral infections. In humans infected with HIV, an imbalance in the Th1 versus the Th2-type responses is thought to contribute to the immune dysregulation associated with progression of disease (Clerici and Shearer 1993). This switch in the predominance of responses associated with Th cells also applies to CD8+ T lymphocytes which can develop a Th2-like function characterised by B lymphocyte helper function and reduced cytolytic activity during infection with this virus (Maggi *et al* 1994).

The reasons for the development of a progressive arthritis in only a proportion of sheep or goats infected with lentiviruses is unclear but it is feasible that it may involve a similar shift in T cell responses. Although Th1 and Th2-type responses have not been identified in ruminants, an attempt to address the issue has been made by Cheevers (1994). PB lymphocytes from non-arthritic CAEV-infected goats stimulated *in vitro* with viral antigen expressed γ-IFN, a characteristic of Th1 responses (Seder and Paul 1994), whereas PB lymphocytes from arthritic CAEV-infected goats did not. Although the production of cytokines characteristic of Th2-

type lymphocytes was not investigated in this study the results suggest that T cell responses to viral antigens may be different in goats that develop arthritis compared to those that do not develop clinical signs.

3. Comparison of synovial tissues from MVV-infected sheep with those from sheep with AIA

Comparison of the histological features of synovium from humans with different arthropathies has revealed that there are very few features that are peculiar to specific disease conditions (Cooper *et al* 1981). Similarly, immunopathological examination of synovial tissues from humans with clinically-distinct inflammatory joint diseases have revealed many similarities in the cell populations present in these tissues and in their expression of activation and other molecules (Lindblad and Hedfors 1983, Kingsley *et al* 1988).

Most of the results described in Chapters 4 and 5 of this thesis relate to the phenotypic characteristics of synovial tissues from sheep infected with MVV or following the generation of AIA. Qualitative comparison of synovium from arthritic MVV-infected sheep with that from the more chronic stages of AIA revealed some differences in basic histological and immunohistological features. A predominance of CD8+ T lymphocytes over the other T lymphocyte subsets was a consistent feature of synovium from arthritic MVV-infected sheep whereas CD4+ T lymphocytes generally predominated in synovia from sheep with AIA. Some of the factors responsible for attracting and regulating the entry of lymphocytes into synovium were discussed in Chapter 1. Very little is known regarding the regulation of entry of specific subsets of lymphocytes into tissues. However, *in vitro* studies have shown that MIP-1α is capable of inducing chemotaxis of activated CD8+ T lymphocytes preferentially over CD4+ T lymphocytes and of promoting adherence of this subset to activated endothelium (Taub *et al* 1993). It is possible that this or other cytokines were responsible for the predominance of this subset in synovia from arthritic MVV-infected sheep.

Although not quantitated, the vascularity of synovia from some arthritic MVV-infected sheep appeared to be considerably greater than that observed in synovia from sheep with AIA. Many of the factors responsible for inducing neovascularisation of tissues such as PDGF, TGFα and TNFα are the products of activated macrophages and fibroblasts (Klagsbrun and D'Amore 1991). Studies of synovial tissues from humans with inflammatory joint disease have shown that macrophage-derived cytokines are produced in abundance compared to those from lymphocytes (Arend and Dyer 1990). On the assumption that this is also true for the arthropathies described in this thesis, differences in the histological appearance of synovia and in the extent of articular cartilage erosion between the two diseases may have resulted from differences in the mechanisms of activation of these cell populations. Different patterns of mononuclear cell infiltrate in synovia have been found to be associated with different cytokine profiles in humans with inflammatory joint disease (Yanni *et al* 1993) and the same is likely to be true for synovial tissues from sheep.

Also, the expression of metalloproteinases in joint tissues has been found to differ significantly between different experimentally-induced arthropathies in rabbits (Henderson *et al* 1990, Hembry *et al* 1993). These mediators play an important role in the destruction of joint tissues (Woolley *et al* 1977). Erosions of articular catilage were a feature of MVV-associated arthritis but not the AIA generated in this study. Qualitative or quantitative differences in the production of metalloproteinases may have played a significant part in these observed differences.

4. AIA in sheep and the investigation of cell trafficking through synovial tissues

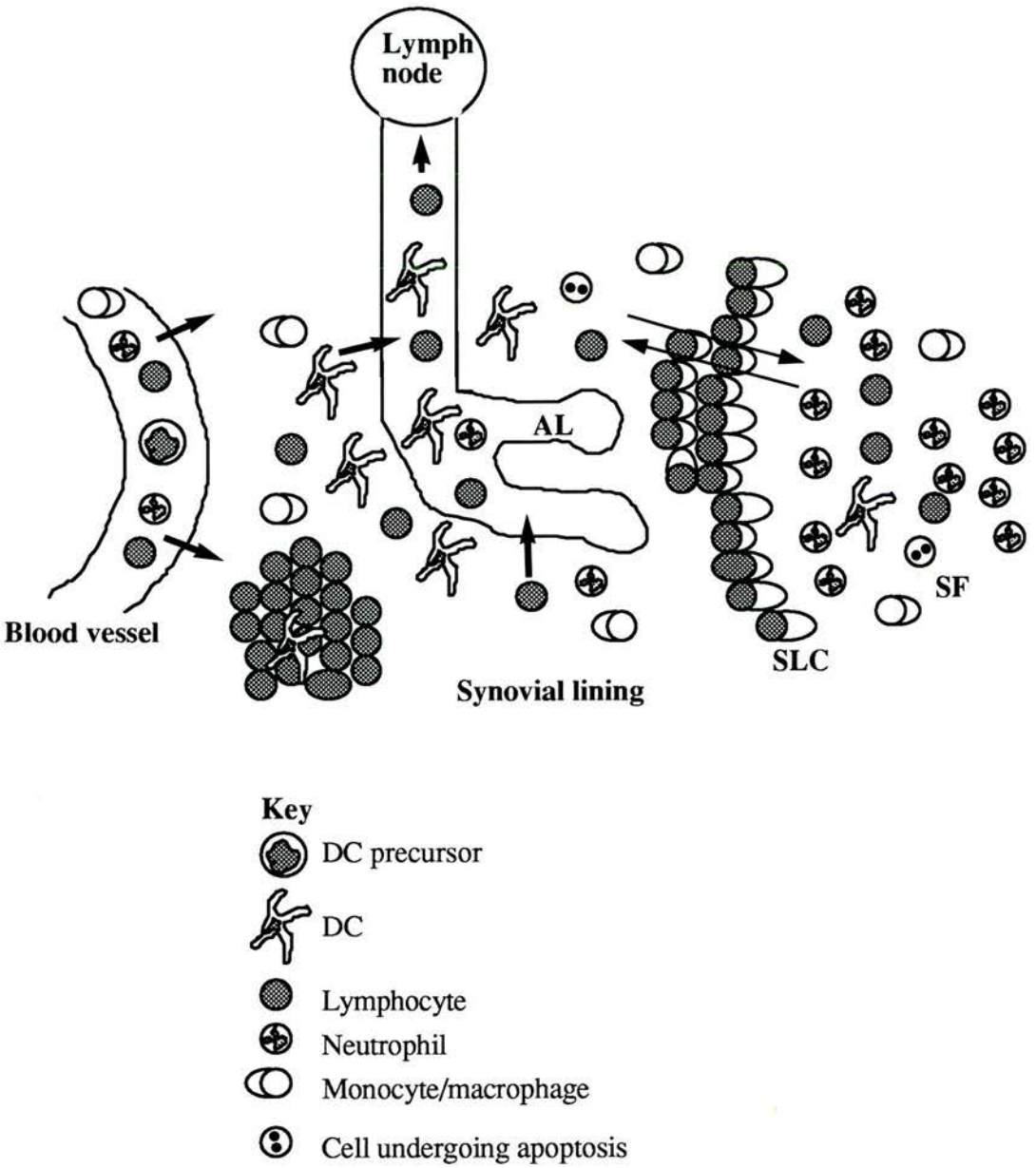
AIA generated in a number of different species has allowed many aspects of the pathophysiology of synovial inflammation to be investigated (reviewed in Chapter 1 and 5). The results obtained in Chapter 5 and 6 of this thesis confirm

that when generated in the sheep AIA also provides a suitable model with which to study the trafficking of cells through joint tissues.

Understanding the characteristics of cell trafficking through the synovial lining and between this tissue and SF is important for a more complete understanding of the biology of normal and inflamed synovium (illustrated schematically in Figure 1). Although it has been established from the investigation of inflammatory arthropathies in humans that the proportion of CD8+ T lymphocytes relative to CD4+ T lymphocytes is higher in SF compared to the synovial lining (reviewed in Henderson and Edwards 1987), our lack of understanding of the dynamics of cell trafficking within and through joints has largely ignored the possibility that different cell populations may have preferential migration pathways through synovial tissues. Some cell types, such as the large basophilic lymphoblasts identified in AL in Chapter 6 were very rarely observed in SF. This implies that these cells may migrate from the synovial lining preferentially via the afferent lymphatic vessels rather than enter SF. This has implications for studies that attempt to establish the degree of clonal homogeneity of the lymphocyte populations in SF.

Understanding the mechanisms involved in the migration of accessory cells into and out of inflamed joints is important from the point of view of antigen handling by joint tissues. Persistence of antigen and its presentation by cells in synovial tissues is regarded as fundamentally important for the perpetuation of chronic synovial inflammation (Klareskog 1991). Although many cell types present in chronically inflamed synovium are capable of presenting antigen, DC are present in abundance and have been identified closely associated with lymphoid cells in the SI (Iguchi *et al* 1986) and DC from SF have been shown to have potent antigen-presenting function *in vitro* (Stagg *et al* 1991). Although it has not been confirmed, these findings suggest that DC may be presenting antigen *in situ*. In spite of the large numbers of cells in inflamed synovial tissues that have the potential to act as APC, the evidence for local activation of lymphocytes either in

Figure 1. Schematic overview of cell trafficking in synovial tissues during inflammatory arthritis



terms of proliferation or cytokine secretion is limited (reviewed in Chapter 1). Although the reasons for this are unclear, one explanation for this may relate to the expression of costimulatory molecules by APC in the joint. The B7 family of proteins (B7-1 and B7-2) are regarded as being the most potent costimulatory molecules involved in the activation of T lymphocytes (Linsley and Ledbetter 1993) and expression of different members of this family can differentially activate Th1 and Th2 lymphocytes (Kuchroo *et al* 1995). Although relatively few studies have investigated the role of these subsets in chronic inflammatory joint disease, Th1 lymphocytes have been found to predominate in the joints of humans with chronic inflammatory arthritis (Simon *et al* 1994). Treatment of mice with flare reactions following AIA with Mab to IL2 and IL4 resulted in a significant degree of diminution of joint swelling with both Mab implying that both Th subsets may play a role in the pathogenesis of flare responses (Jacobs *et al* 1994b). Because mast cells are also a source of IL4 (Schwartz 1993) the possibility could not be ruled out that treatment with this Mab was affecting this cell population and not IL4-producing T lymphocytes. Several studies have shown that B7 expression by macrophages and DC in chronically inflamed synovial tissues is weak or absent (Munro *et al* 1994, Summers *et al* 1995) suggesting that either alternative costimulatory molecules are utilised or that their expression has been inhibited or downregulated. B7-CD28 interactions are also indirectly required for B cell responses by controlling Th cell cytokine secretion (Linsley and Ledbetter 1993). The low levels of B7 expression in synovium may serve to limit local antigen-specific antibody and autoantibody production.

Very little is known of the rate of turnover of DC in normal or inflamed tissues. If DC with captured antigen were retained in inflamed synovial tissues in a similar fashion to that suggested for LC in the skin of humans with psoriasis (Streilein *et al* 1990) this may provide a mechanism for the perpetuation of chronic inflammation in these tissues. Cytokines have been implicated in the migration of LC from the skin to the local draining lymph node (Cumberbatch and Kimber

1992). Given the likelihood that multiple cytokines may play a role in this process and that the balance of these cytokines may change during the course of an inflammatory response, the tendency of these cells to migrate or be retained *in situ* may alter during an immune response. DC that do acquire antigen and migrate from the joint could initiate T cell responses in the draining lymph node. Thus the kinetics of DC turnover in inflamed synovial tissues may be particularly relevant for understanding antigen handling by these tissues. .

Understanding the kinetics of lymphoid cell turnover in chronically inflamed synovial tissues is also of importance. The turnover of lymphoid cells in a chronically inflamed joint will be influenced by a number of factors including persistence of antigen, the lifespan of different lymphoid cell populations and their antigenic specificities, the maintenance of immunological memory and possibly the predisposition of some cells to recirculate preferentially through the inflamed joint. Antigen-specific and antigen non-specific B and T lymphocytes will migrate into the joint during AIA and following induction of flare reactions. The results shown in Chapter 6 of this thesis show that a proportion of these lymphoid cells enter the AL draining the joint. An unresolved issue is whether antigen-specific T lymphocytes are retained preferentially in the joint during these responses, and if so for how long. Trapping of antigen-reactive cells following administration of antigen has been confirmed in lymph nodes and spleen (Hopkins *et al* 1981c) and given the long-term persistence of antigen in the joint it would seem likely that this would also be the case for synovial tissues. For those cells that are retained in the joint or that develop a predisposition to recirculate through the inflamed tissues, the long average life-span of memory cells (Gray 1993) will favour perpetuation of chronic inflammation. Although it has been established that lymphocytes draining from chronically inflamed skin have a predisposition to recirculate through these tissues (Chin and Hay 1980), it is not known for how long this phenomenon persists for individual cells or whether these cells are specific for the inciting antigen. Repeated antigenic stimulation of cells is thought to be of importance in

the maintenance of immunological memory (Gray 1993) and may possibly play a role in maintaining preferential lymphocyte migration pathways.

The accumulation of moderate numbers of plasma cells was a feature of synovial tissues from sheep with AIA (results Chapter 5). Whether antigen-specific B lymphocytes that traffic through the inflamed joint receive the appropriate signals to differentiate into plasma cells will depend upon a number of factors including the presence of antigen-specific T lymphocytes and the local concentrations of antigen-specific antibody and antigen. An excess of antigen-specific antibody relative to antigen resulting in IC forming in antibody excess will inhibit the differentiation of antigen-reactive B lymphocytes into plasma cells by the cross-linking of antigen receptors with FcγRII on the surface of the B cell (Ravetch 1994). Under these circumstances B lymphocytes will traffic through the joint until such time as the antigen:antibody ratio optimises to allow B cell differentiation. The life-span of plasma cells that have differentiated locally in the joint or that are derived from activation of B cells in the draining lymph node is not known. Short and long-lived antibody-producing cell populations have been identified (Ho *et al* 1986) and the proportions of these cells vary at different sites in the body and arise from B cell activation under different conditions (Ho *et al* 1986). Antigen-specific B lymphocytes that localise to the joint that have been activated by follicular DC in the draining lymph node are likely to be long-lived cells (ie in excess of 3 weeks). However the relative numbers of B cells that are activated locally in the joint versus those that are activated in the draining lymph node is not known.

Future prospects

There are many aspects of the biology of normal and diseased synovial tissues that remain unclear. To date, most of our understanding of the disease processes affecting these tissues has been obtained from studies of human synovial tissues. Sheep have a significant advantage over laboratory species that are used to study the characteristics of synovial immune responses in terms of the increased quantity

of synovial lining and SF that are available at all stages of development. The major disadvantage of using this species are the smaller numbers of animals that can be used in experimental studies.

All animal models of chronic inflammatory joint disease present opportunities for different components of this disease process to be investigated. The potential to perform lymphatic cannulation studies in sheep allows some features of synovial immune responses to be investigated that cannot be addressed in other species. The observations made in Chapter 6 of this thesis need to be extended with larger numbers of animals but they suggest areas for further research. Very little is known of the factors that are responsible for retention of different cell types in chronically inflamed synovium. Elucidating the differences in phenotype of cells in the AL draining an inflamed joint and those that are retained in the synovial tissues allows conclusions to be drawn regarding factors that may play a role in their retention within this enviroment. With the increasing availability of recombinant ovine cytokines (McInnes 1993) the factors that may be responsible for emigration of cells from inflamed synovium could be investigated.

To address the issue of whether specific populations of T lymphocytes are preferentially expanded or retained within the joints of humans with inflammatory arthropathies the frequencies of T lymphocytes bearing particular V genes have been determined (Cooper *et al* 1991, Andreu *et al* 1991). Comparing the frequencies of antigen-specific T and B cells in the joint during progression of AIA with those in AL at different time points following antigenic challenge would establish the kinetics of trafficking of antigen-reactive lymphocytes through inflamed joints. If this were established for a disease where the inciting antigen is known, such as AIA, it may shed light upon investigations performed on inflammatory arthropathies in humans whose aetiologies are often poorly understood.

The role of antigen carriage by different cell populations from an inflamed joint could be established by functional assays using OVA-specific T cell lines or

clones that have previously been generated in the sheep (Bujdoso *et al* 1989c). This would be a sensitive technique for determining the kinetics of antigen clearance by cells emigrating from an inflamed joint.

The question of whether lymphocytes have a predisposition to recirculate through a chronically inflamed joint could be addressed by cell-labelling studies provided afferent lymphatics draining different tissues were cannulated simultaneously in the same sheep, in a similar fashion to previous studies that have investigated this phenomenon at other anatomical sites (MacKay *et al* 1992b). If lymphocytes that emigrate from an inflamed joint have a tendency to recirculate through these tissues it would provide a further mechanism whereby chronic inflammation in joints could persist.

The investigations described above have the potential to be applied to other inflammatory arthropathies of sheep including MVV-associated arthritis. Thus the sheep as an experimental animal provides unique opportunities for research into basic pathophysiological mechanisms underlying chronic inflammatory joint disease.

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Quantitative Analysis of Immunohistological Changes in the Synovial Membrane of Sheep Infected with Maedi–Visna Virus

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Ve have carried out a quantitative immunohistological alysis of synovial membrane from the joints of clinically hritic sheep naturally infected with Maedi–Visna virus VV) and compared the results to subclinically affected nts (carpal and tarsal) from infected sheep and to joints m a control population. Significantly elevated numbers of three T lymphocyte subsets (CD4+, CD8+ and γδ) were nd in the synovia from clinically arthritic sheep com-ed to controls. There was also a significant increase in number of CD8+ T lymphocytes in the carpal synovium subclinically arthritic animals. In both clinically ar-itic and subclinical disease states CD8+ T cells predom-ted over CD4+ T cells and T cells bearing the γδ T cell eptor. Significant increases were also observed in the mbers of cells staining for MHC class II antigens in the ovial lining cell layer and subintimal cell populations of ovia from clinically arthritic sheep. These increases were parent in the subintimal cell population at the subclinical ge of disease. Macrophage-like cells staining for the viral e protein p15 were observed in some of the most inflamed mples. The data are thus consistent with a disease process ven by chronic viral antigen presentation to infiltrating T ls, and could serve as a model for elucidating the mech-sms underlying some types of inflammatory joint disease man.

INTRODUCTION

Maedi–visna virus (MVV) is a nononcogenic lentivi-s that causes chronic inflammatory disease in pri-arily the lung (Maedi) and nervous system (Visna) d to a lesser extent the mammary gland and joints of eep (1, 2). DNA hybridization studies and sequence mology have shown a close relationship between VV and caprine arthritis–encephalitis virus (CAEV) at infects goats (3, 4). Both viruses can cause a ronic progressive synovitis that in a proportion of dividuals results in joint destruction (5–8). The dis-se process is characterized by a long incubation pe-d that may last months to years followed by the slow velopment of clinical signs (6, 9). Histologically the novitis is characterized by infiltration with macro-ages (mϕ) and accumulation of lymphoid cells which advanced cases can resemble lymphoid follicles (10, 11). The histological appearance of synovium and clinical features have drawn comparison with rheumatoid arthritis in man although to date detailed immunohistological information has been lacking.

Increasing numbers of reports have revealed that the human immunodeficiency virus (HIV), a lentivirus related to MVV and CAEV, may play a role in the development of a variety of arthritic syndromes in man (12). HIV has been isolated from dendritic cells (DC) in synovial fluid and synovial membrane changes have been reported in AIDS victims at postmortem (13, 14). The role of HIV in the initiation and perpetuation of these changes has not been established but in MVV and CAEV infection lesions are believed to be induced primarily by the interaction between viral antigens and the host immune responses (15–17).

In MVV and CAEV the main joint to be affected clinically is the carpus with the tarsal joints being affected to a lesser degree. Previously we have documented changes in the cellular composition of synovial fluid from MVV-infected sheep with subclinical arthritis compared to controls (18). In particular the mϕ/DC cell populations showed marked increases in the expression of major histocompatibility complex (MHC) class II antigens, while the lymphocytic infiltrate showed a predominance of CD8+ over CD4+ T cells or T cells bearing the γδ T cell receptor. The present study was aimed at characterizing in detail the immunohistochemical profile in the synovium of MVV-infected sheep that were clinically arthritic and comparing the changes both to subclinically affected joints and to the joints of a control population of sheep.

METHODS

Sheep and Synovial Membrane Preparation

Synovial membrane was obtained from the affected joints of 6 clinically arthritic sheep naturally infected with MVV. These were identified by the presence of gross periarticular soft tissue thickening +/− lameness. In 4 cases the carpal joints were affected (2 bilaterally, 2 unilaterally) and the remaining 2 had bilaterally affected stifle and elbow joints, respectively.

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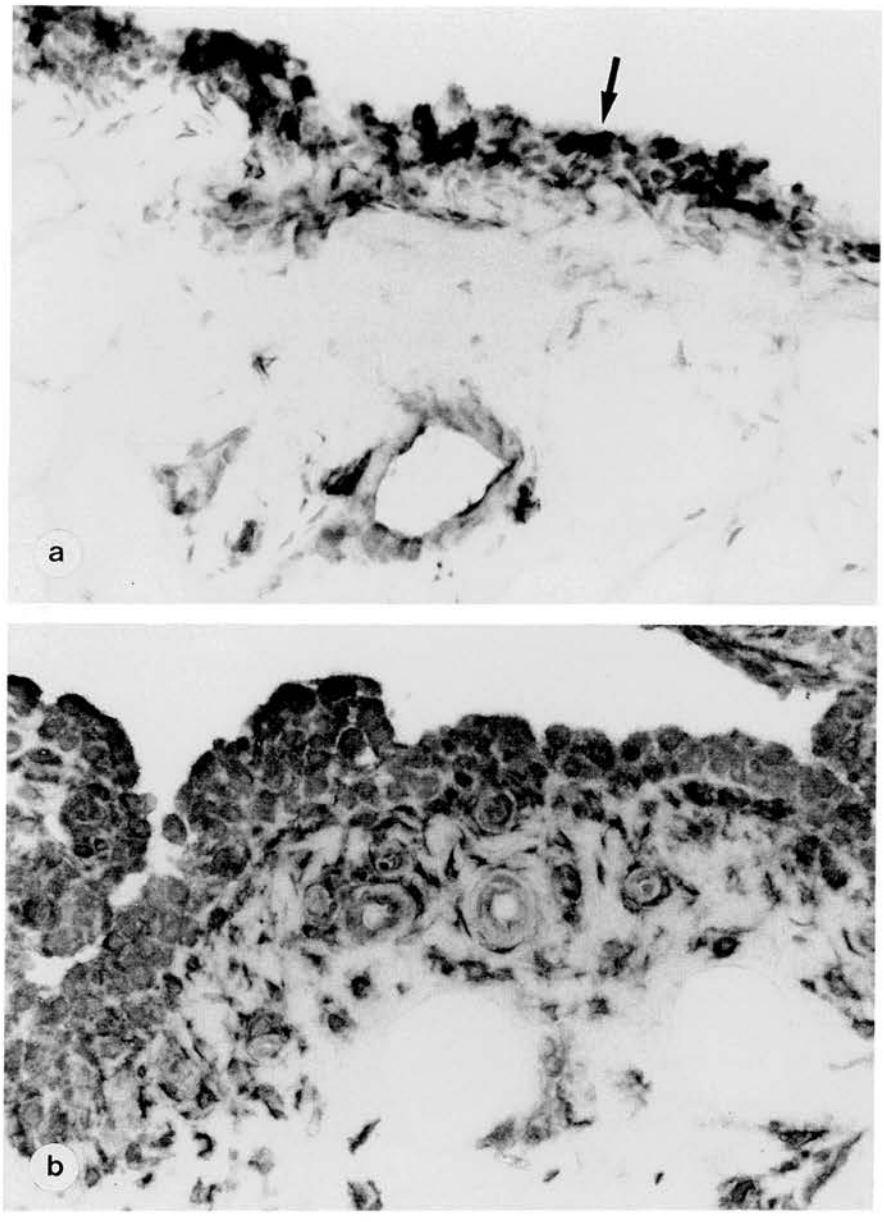

FIG. 1. Carpal synovial membrane from control (a, c) and clinically arthritic MVV-infected sheep (b, d) stained with Mab to pan M class II. Immunoperoxidase technique, magnification ×320. Representative positive cells in the lining layer and subintimal tissue indicated with arrows in the control sections. Virtually all of the cells in the lining layer and subintimal tissue from infected sheep positive.

MVV infection was confirmed serologically by a standard agar gel immunodiffusion test (AGID). In all cases tissue was obtained at postmortem. The reason for euthanasia was progressive worsening of clinical status in terms of severe weight loss, dyspnea, or lameness. Synovial membrane was also obtained from the right radio-carpal and right tibio-tarsal joints from 8 sheep naturally infected with MVV but with no evidence of clinical arthritis. This was obtained in 4 cases at postmortem and in the remaining 4 cases at open arthrotomy under general anesthesia (intravenous alphadolone/alphaxalone- "Saffan," Pitman-Moore, Middlesex, UK) followed by a balanced mixture of halothane, oxygen, and nitrous oxide). All sheep were fema aged over 4 years, and either of the Texel (13 anima or Blau de Maine (1 animal) breed. Ten age- and s matched control sheep were obtained from a flock w no history of MVV infection (and were seronegative AGID). Breeds with similar size and conformation Texels were chosen. These included 7 Cheviots, 1 D set Horn cross, 1 Suffolk, and 1 Texel. Synovial me brane was obtained at arthrotomy in the way describ from the right radio-carpal and right tibio-tarsal join None had shown clinical signs of joint disease prior biopsy. A portion of synovium was placed in form saline for routine histopathological processing wh

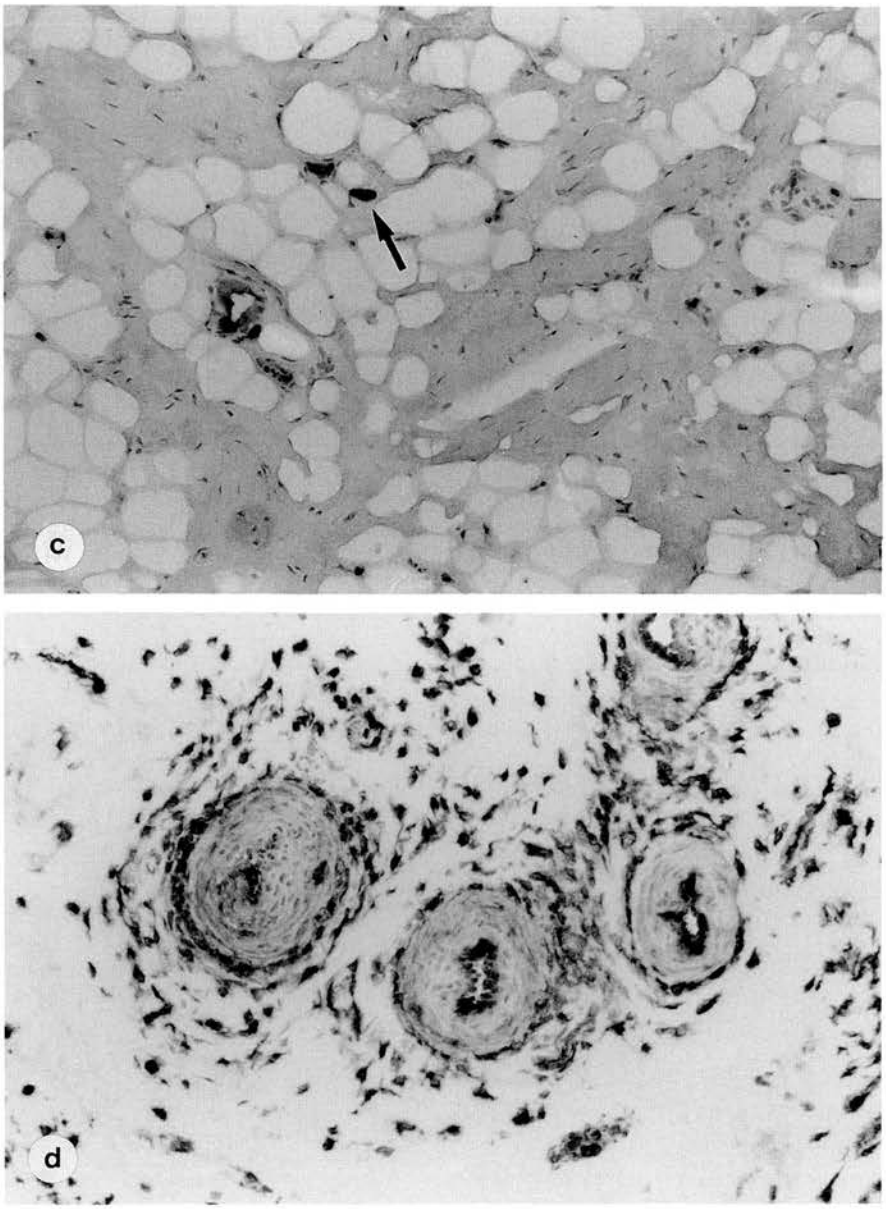

FIG. 1—*Continued*

remainder was coated in OCT compound (Miles entific, Naperville, IL) and snap-frozen in dry ice/ pentane, then stored at −70°C.

nunocytochemistry

rozen sections of synovium 6- to 8-μm thick were ced on slides coated with D-lysine (Vectabond), air- ed a minimum of 2 hr, then fixed in acetone (4°C) for in. Prior to the application of monoclonal antibod- (Mab) nonspecific binding was blocked by incubat- the sections in diluted bovine serum albumin (2%), mal rabbit serum (2%), and normal sheep serum) for 20 min. Mabs applied to the sections were cific for ovine CD4 (SBU-T4), CD8 (SBU-T8), γδ T s (86D), pan MHC class II (combination of Mabs covering all known epitopes), DRβ (VPM37), and DQβ (VPM41) (Refs. 19–21). In addition a Mab specific for the viral core protein P15 (22) was used. Control sections were incubated in irrelevant isotype-specific Mabs. The sections were incubated overnight at 4°C. Following several washings in phosphate-buffered saline a streptavidin–biotin-peroxidase kit (Immustain, Euro DPC, Witney, UK) was used to demonstrate the aforementioned antigens. Endogenous peroxidase was blocked by glucose oxidase immediately prior to application of 3′,3-diaminobenzamine substrate. The sections were lightly counterstained with hematoxylin.

Cell Quantitation

Using a graticle field, 500 synovial lining cells were counted in identical areas of sequential sections for

Mabs pan MHC class II, VPM 37, and VPM 41. Expression of these antigens was determined in a similar fashion for cells within the subsynovium (500 cells counted). T lymphocyte numbers were determined by counting positive cells in identical areas of synovium on sequential sections. A minimum area of 3 mm^2 (range 3–5 mm^2) of tissue was counted. The results were analyzed using the Mann–Whitney nonparametric rank test.

RESULTS

Synovial Histology

Histological evidence of chronic synovitis was present in all tissue samples from clinically arthritic MVV-infected animals. The changes were characterized by extensive lymphoid infiltrates with focal and perivascular aggregates, macrophage infiltration, vascular proliferation, and the replacement of subintimal adipose tissue with fibrous tissue. Moderate numbers of plasma cells were seen in 3/6 tissues and moderate synovial lining cell hyperplasia (3–6 cells thick) was seen in 2/6 samples. Similar changes were observed in synovia from infected animals not showing clinical signs, though the degree of infiltration and intensity of tissue changes were considerably less. Tissues from three clinically arthritic and three subclinically affected animals had small arteriolar vessels with massive smooth muscle hyperplasia of their walls and endothelial cell swelling (Fig. 1d). Usually only a few such vessels were seen in each section, but these changes were never seen in control synovia.

Expression of MHC Class II Molecules

The numbers of synovial lining cells (SLC) expressing MHC class II molecules were significantly elevated in clinically arthritic sheep compared to controls tissues ($P < 0.001$ for pan reagent, and DR and DQ-specific reagents) (Figs. 1a, 1b, 2). No significant difference was found between the numbers of SLC expressing the DRβ or DQβ subsets. In normal synovia, only a proportion of the most superficial SLC expressed MHC class II (Fig. 1a), whereas the vast majority of SLC at all depths were positive from clinically affected animals (Fig. 1b). Intermediate numbers of MHC class II expressing SLC were observed in synovia from subclinically affected animals (Fig. 2). The differences between clinical and subclinical states and between subclinical and normal animals, however, were not statistically significant. Also, although the mean values obtained from the tarsal joints of subclinically arthritic and normal animals were lower than the corresponding values from the carpal joints, the differences were not statistically significant.

A significant increase in MHC class II expressing subintimal cells (SIC) was observed between clinically arthritic joints and normal carpal tissues ($P < 0.002$ for

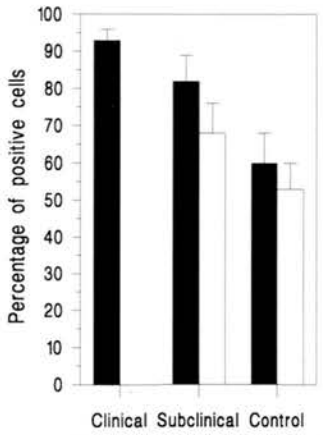

FIG. 2. Expression of MHC class II by synovial lining cells. represent mean ± SEM for the pan reagent (n = 6, 8, and 10 fo clinical, subclinical, and control groups, respectively). For subc cal and control groups, solid bars represent carpal synovia and bars represent tarsal synovia. There was no statistically signifi difference between the staining observed for pan MHC class II, and DQ-specific Mabs within any group.

pan reagent, DR and DQ-specific reagents) (Figs. 1d, 3). No significant difference was found between numbers of SIC expressing DRβ or DQβ. The numb of MHC class II expressing SIC in synovia from s clinically arthritic animals were elevated over con samples ($P < 0.008$ for pan reagent, and DR and subset-specific reagents), but also significantly lo than in tissues from clinically affected animals (P 0.04 for all three reagents) (Fig. 3). The differer between the carpi and tarsi in subclinically affec and normal tissues were not statistically significar

The morphology of the MHC class II+ SIC res bled lymphocytes, macrophages, and fibroblasts (1d). Many of the endothelial lining cells of blood

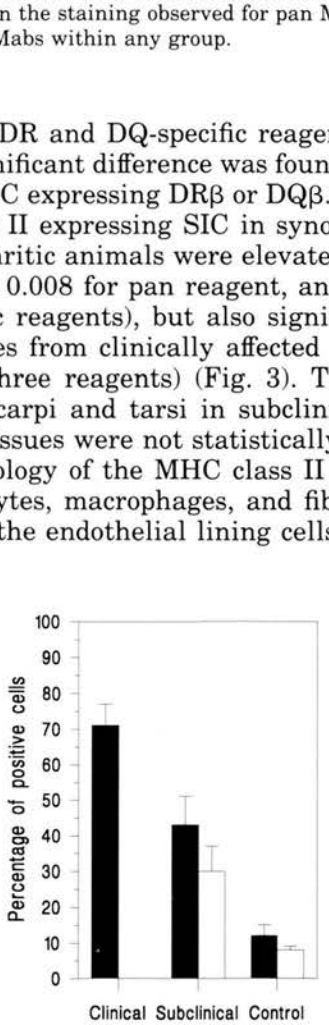

FIG. 3. Expression of MHC class II by subintimal cells. represent mean ± SEM for the pan reagent (n = 6, 8, and 10 fo clinical, subclinical, and control groups, respectively). For subc cal and control groups, solid bars represent carpal synovia and bars represent tarsal synovia. There was no statistically signifi difference between the staining observed for pan MHC class II, and DQ-specific Mabs within any group.

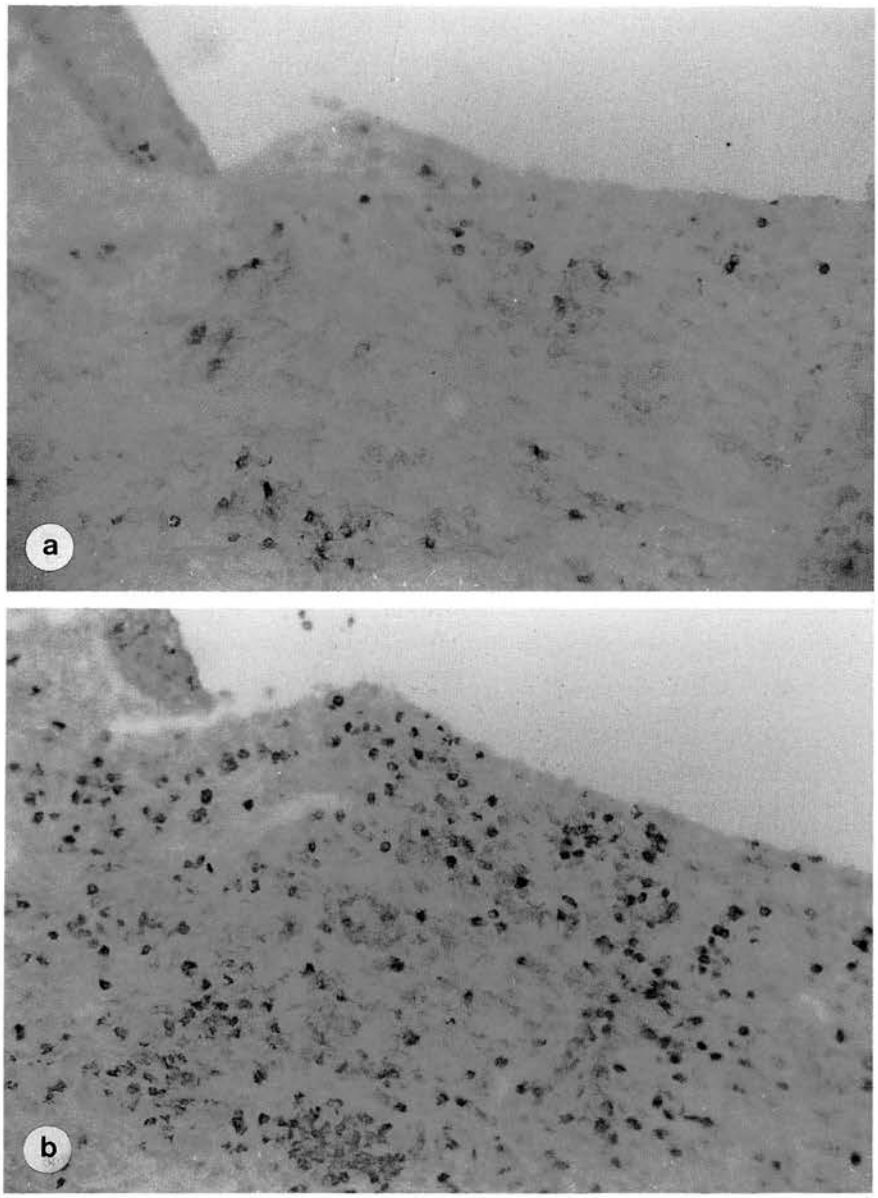

FIG. 4. Carpal synovial membrane from clinically arthritic MVV-infected sheep stained with Mabs to ovine CD4+ (a), CD8+ (b and d) d γδ T cell receptor-bearing lymphocytes (c). Immunoperoxidase technique, magnification ×100.

ıs were positive (Fig. 1d), but these could not be antitated accurately.

Lymphocyte Subsets

The densities of all T lymphocyte subsets in synovia om clinically affected animals were significantly ised compared to control tissues ($P < 0.004$ for all ree subsets) (Figs. 4a–4d, 5) which were usually arsely infiltrated. However, the ratios of CD8:CD4 d αβ:γδ did not differ from control samples. When bclinical carpal tissues were compared to carpal con-ols the only T subset whose density was significantly evated was CD8+ ($P < 0.04$) (Fig. 5). No significant ferences were present between subclinical and control tarsal tissues. Most of the CD8+ T cells were diffusely scattered in bands immediately below the synovial lining cells (Fig. 4b). In tissues with extensive lymphoid infiltrates focal and perivascular aggregates of CD8+ T cells were also seen deeper within the subintimal tissue (Fig. 4d). Although the numbers of γδ T cells relative to the number of αβ T cells were generally small (Fig. 4c), in two samples they constituted 36 and 35% (one clinical and one subclinically affected animal, respectively) of the total T cell infiltrate. They were found diffusely scattered throughout the subsynovium and generally showed no tendency to aggregate.

Occasional P15+ cells were observed in tissues from affected carpal and stifle joints from three clinically affected sheep (Fig. 6). These cells morphologically re-

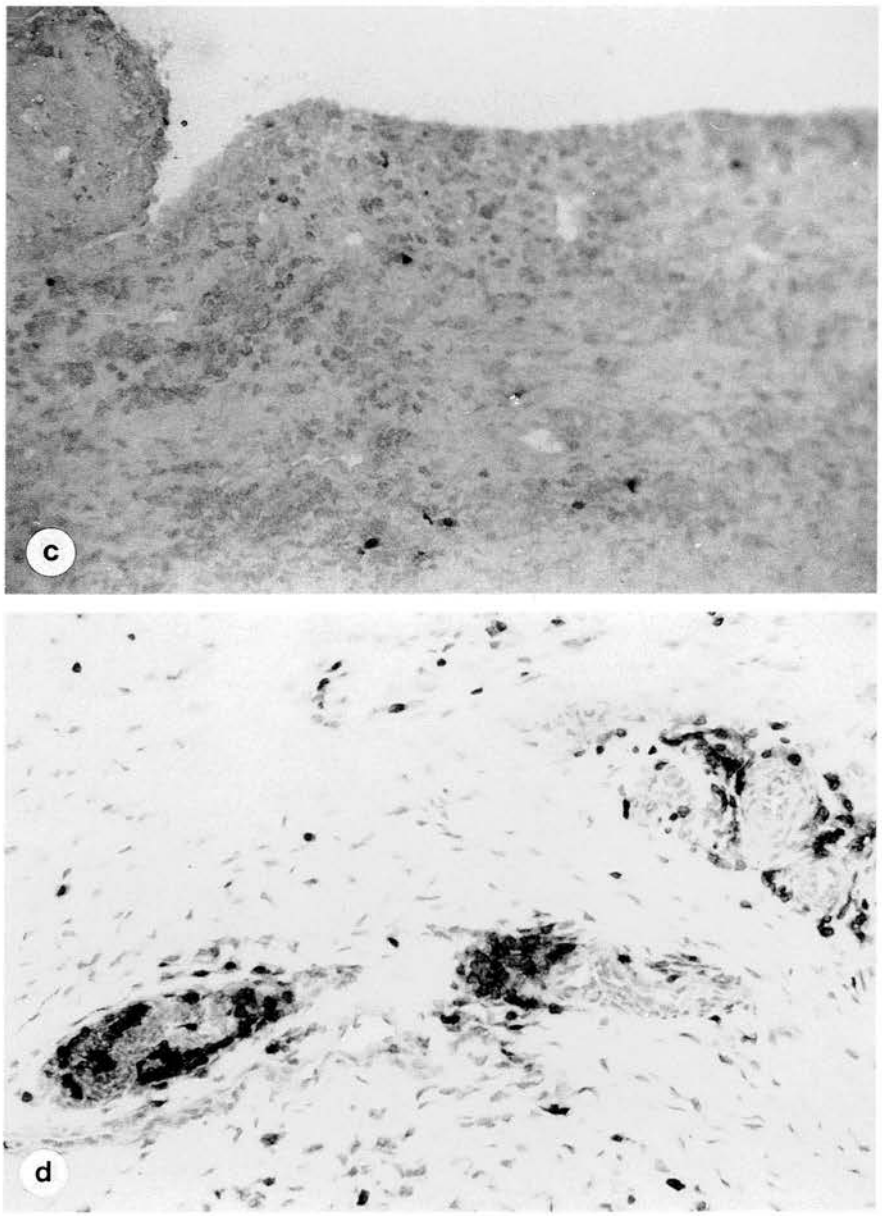

FIG. 4—*Continued*

sembled macrophages and were located in the subintimal tissue.

DISCUSSION

MVV- and CAEV-associated arthritis provide natural models of chronic inflammatory arthritis where a defined etiological agent is believed to play an important role in the pathogenesis of the disease. Previous investigations have largely concentrated on CAEV infection because of the predominant tropism of this agent for synovial tissue in adult animals, although detailed immunohistological information is lacking for both diseases. This study aimed to provide detailed immunohistological data for synovia from normal a mals and from sheep infected with MVV that were b clinically and subclinically affected.

The 6 animals described here were the only clinica arthritic sheep to be seen over a 3-year period fro flock of approximately 90 naturally-infected anima This low incidence of clinical disease is in accorda with previous studies of naturally infected flocks.

The most noticeable histological difference betwe tissues from clinically arthritic animals and tiss from subclinically affected sheep was the degree of ly phoid infiltration. Most of the cells in tissues from s clinically affected sheep resembled mϕ and fibroblas Increased levels of fibrosis and vascularity were co mon, however, and were present to a greater extent

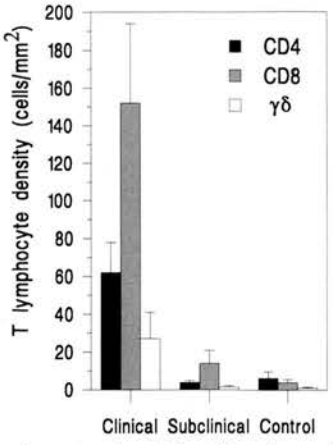

FIG. 5. T lymphocyte subset densities from clinically arthritic, clinical, and control sheep. Bars represent mean ± SEM (n = 6, and 10 for clinical, subclinical, and control groups, respectively). subclinical and control groups, only values from the carpal sy- ia are shown. There was no statistically significant difference ween the densities of the T cell subsets from carpal and tarsal ts within subclinical and control groups.

ose tissues with extensive mϕ infiltrates. A large portion of these cells was activated as judged by pression of MHC class II molecules and activated mϕ ve previously been shown to be potent inducers of scular proliferation (23, 24). A histological feature t apparently observed in previous studies was the ooth muscle hyperplasia present in a proportion of e smaller arterioles. These changes together with as- iated endothelial cell swelling very closely resemble teriolar changes that have been observed in synovia m AIDS patients (13). The authors of the latter re- rt suggested that these changes may have been the cause of the fibrosis and loss or thinning of the lining cells that they had observed. Although fibrosis was a feature of the synovium in these animals similar changes in the lining cell layer were not observed. Similar blood vessel changes have also been reported from the lungs and other parenchymal organs of macaques infected with simian immunodeficiency virus (SIV) (25) and human pediatric AIDS patients (26). The etiology of these blood vessel changes at present is not known.

An increased expression of MHC class II antigens in a variety of tissues has been reported following experimental infection with MVV (27). This is believed to be the result of interaction between virally infected mϕ and T lymphocytes with the production of interferon-like molecules (28). It is possible that the elevated expression of MHC class II by the lining cells and subintimal cells in tissues from infected animals is due to the *in vivo* production of these or other cytokines (29). In this respect the observation of significantly increased numbers of CD8+ T cells in subclinically affected carpal tissues compared to controls was of particular interest. Previously we have reported similar elevated MHC class II expression by the mϕ/DC population in synovial fluid of MVV infected sheep (18). The observation may, in part, explain the exaggerated inflammatory response observed on generation of an antigen-induced arthritis in goats infected with CAEV (30) considering that surface expression of MHC class II correlates well with antigen-presenting capacity in this cell type (31). Although the differences were not statistically significant, carpal synovia from subclinically affected sheep did show increased numbers of CD8+ T cells and MHC class II+ subintimal cells

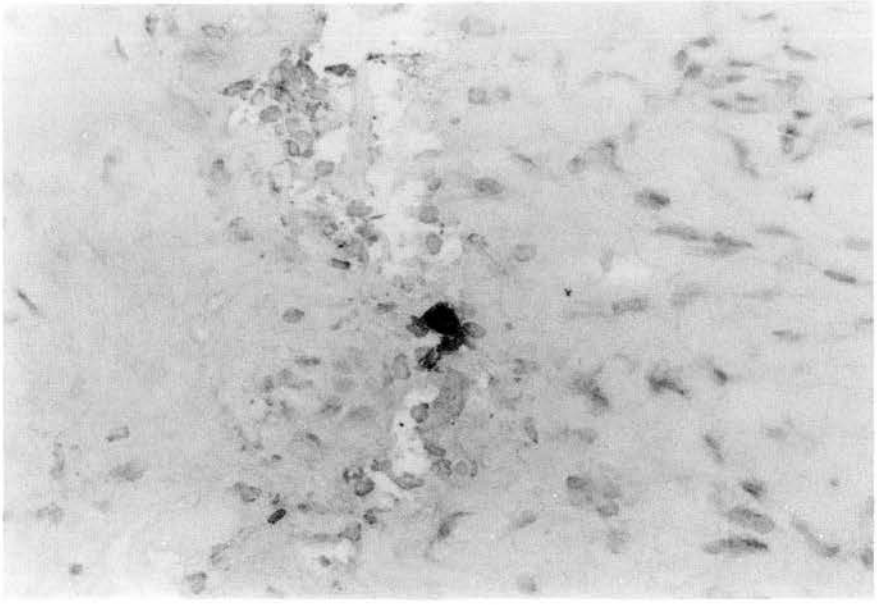

FIG. 6. Synovium from stifle joint of a clinically arthritic MVV-infected sheep stained with Mab to P15 viral core protein. Immunoperdase technique, magnification ×320.

compared to tarsal tissues from the same animals. Whether this predisposes the carpal joints to disease progression remains to be established.

Paucity of viral antigen as detectable by immunocytochemistry has been observed previously in CAEV-associated arthritis (9, 11) and in HIV-associated arthropathies (32) but recovery of virus from synovial tissue does generally correlate with lesion severity (9–11, 33). Virus isolation was successfully performed from the synovia from clinically arthritic sheep (data not shown). Recently the application of *in situ* PCR techniques to tissue from sheep experimentally infected with MVV has shown very large numbers of cells to be harboring proviral DNA compared to the numbers with detectable levels of mRNA in areas of inflammation using standard *in situ* hybridization techniques (34). It is possible that some of these cells may be expressing viral antigen at levels undetectable by current immunohistological methods. The number of viral protein-expressing cells as determined here may thus be a gross underestimate of the virus load within the tissue.

In the majority of normal sheep CD8+ T lymphocytes predominate in the synovial fluid from carpal and tarsal joints (18). The proportions of these cells and γδ T lymphocytes increases in MVV infection when arthritis is subclinical (18). The present results show that there is a predominance of CD8+ T cells over CD4+ T cells in the carpal and tarsal synovium at all stages of disease. Although the CD8:CD4 ratios were not significantly altered compared to control synovia, the highest ratios were observed in clinically affected animals, suggesting that selective accumulation of CD8+ T cells is associated with disease progression. Recent reports of inflammatory arthropathies associated with SIV infection in rhesus monkeys and HIV in man demonstrated that CD8+ T cells were the dominant T cell subset in synovial tissue (25, 32). The significance of the CD8+ T lymphocyte predominance in the synovial membrane remains unclear. Recent studies have demonstrated the presence of specific anti viral cytotoxic T cell precursors in blood and efferent lymph of sheep experimentally infected with MVV (35). However, whether the CD8+ T cells in the joint have a cytotoxic function in the elimination of virally infected cells remains to be elucidated. Recently it has been shown that the mϕ product MIP-1β can induce preferential migration of activated CD8+ T cells (36). Given that large numbers of activated mϕ are present in the synovia of these animals it is possible that they may release this or similar products that allow selective accumulation of CD8+ T cells. Similarly the function of the γδ T lymphocytes remains unclear although they have been reported from human rheumatoid arthritis and may contribute to the pathogenesis of chronic synovitis (37).

This study has shown that significant immunopathogenic changes occur in some joints of sheep infected with MVV prior to or in the absence of the appearar of clinical disease. Given the potential role of HIV the development of arthropathies in man and the h tological and immunocytochemical similarities in novium between the two diseases, MVV-associated novitis represents a potentially valuable model of h man retroviral-induced joint disease.

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